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Development of a coaxial fiber membrane reactor with immobilized horseradish peroxidase

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Aos meus Pais.
À minha Família.

"Science never solves a problem without creating ten more."
by George Bernard Shaw.

Agradecimentos

"Apenas quando somos instruídos pela realidade é que podemos mudá-la."
por Bertolt Brecht

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Abstract

Fiber membranes obtained by electrospinning are a powerful tool in different areas. One of the ways to broaden the potential of these membranes is to functionalize them through the immobilization of biological species. Encapsulation within fiber membranes offers a protective microenvironment for enzymes, with a high surface area for the exchange of compounds involved in catalytic processes, and may promote enzyme activity even in less favorable environments. The objective of this work was to make coaxial fibers with an inert silica shell and a core that was suited for the immobilization of an oxido-reductase.

The shell was prepared using the sol-gel process. This process is complex. Studies were made varying the silica/water/acid molar ratio, the silica precursor (tetramethoxysilane, TMOS, or tetraethoxysilane, TEOS), the amount of polyvinyl alcohol, which lends flexibility and porosity to the fibers, and also other compounds such as acetic acid, citric acid, methanol and ethanol, in addition to experimental parameters such as time and temperature, time of heating before adding species. Regular fibers with a narrow diameter distribution were obtained by using a 1:2:1.1:8E^{-3}:3.5E^{-4} TMOS/water/ methanol/HCl/PVA molar ratio.

For the fiber cores, the choice was the "Ion jelly", an ionic gelatin formed through cross-linking of gelatin and ionic liquid (IL) molecules. The gelatin was used in low concentration in order to keep the solution fluid for longer times, and the temperature of the electrospinning chamber was kept lower to slow down the solidification of the sol-gel solution. The most used IL in these assays was choline dihydrogenphosphate. This gelatin/IL combination was tested to make films within which the enzyme horseradish peroxidase (HRP) was immobilized. It was found that the enzyme exhibited good activity in films prepared with 10% gelatin and 10% IL (w/w).

The fibers obtained were characterized using several techniques, such as scanning electron microscopy, energy dispersive X-ray spectroscopy, Fourier transform infra-red spectroscopy, electrical conductivity measurements.

In addition to studying experimental conditions that were adequate for preparing each solution, it was also necessary to study the best conditions to combine the two solutions in coaxial electrospinning assays. It was possible to make coaxial fibers that were shown to have a shell rich in silicon and a core rich in phosphorous and sodium. Coaxial fibers with immobilized enzyme were also made, but they revealed no evidence of enzymatic activity. One possible reason for this fact is the low amount of membrane produced, which results in too low an amount of enzyme. It was difficult to control the temperature and humidity in the electrospinning chamber, but by controlling these variables better, it is believed that the formulations developed will allow the fulfilment of the goal to make a coaxial fiber membrane with immobilized HRP for bioremediation.

Keywords: Bioremediation, coaxial, electrospinning, fibers, gelatin, ionic liquid, biocatalytic membranes, oxido-reductase, silica.

Resumo

As membranas de fibras produzidas por electrospinning são uma poderosa ferramenta em diversas áreas. Umas das formas de potenciar as aplicações destas membranas é funcionalizando-as através da imobilização de espécies biológicas. O encapsulamento em membranas de fibras proporciona um ambiente protetor para enzimas, com elevada área superficial para as trocas necessárias à ação catalítica, podendo potenciar a atividade destas mesmo em ambientes mais hostis. O objetivo neste trabalho era fazer fibras co-axiais com um revestimento inerte de sílica, e com um interior propício à imobilização de uma oxido-redutase.

Utilizou-se o processo sol-gel para o revestimento. O processo sol-gel é complexo. Variou-se a razão molar sílica/água/ácido, o precursor de sílica (tetrametoxisilano, TMOS, ou tetraetoxisilano, TEOS), o teor de álcool polivinílico, que confere flexibilidade e porosidade às fibras, e ainda outros compostos, como ácido acético, ácido cítrico, metanol e etanol, para além de condições experimentais como tempo e temperatura, tempo de aquecimento antes de juntar aditivos. Obtiveram-se fibras regulares e com pouca dispersão de diâmetros quando se utilizou uma razão molar 1:2:1.1:8E^{-3}:3.5E^{-4} de TMOS/água/metanol/HCl/PVA.

Para o interior das fibras, opou-se por "Ion jelly", uma gelatina iónica resultante do estabelecimento de ligações cruzadas entre moléculas de gelatina e líquido iónico (IL). Utilizou-se uma gelatina em baixa concentração de forma a permitir manter a solução fluida durante mais tempo, a uma temperatura menos elevada da câmara de electrospinning, para não acelerar o processo de solidificação do sol-gel. O IL mais utilizado foi o dihidrogenofosfato de colina. Testou-se esta combinação em filmes nos quais se imobilizou a enzima peroxidase de rábano (HRP). Verificou-se que a enzima exibia boa atividade em filmes preparados com 10% de gelatina e 10% de IL (p/p).

As fibras obtidas foram caracterizadas por várias técnicas, como microscopia eletrónica de varrimento, espectroscopia de raios-X, espectroscopia de infra-vermelhos de transformada de Fourier, medidas de condutividade elétrica.

Para além de estudar as condições experimentais adequadas à preparação de cada solução, foi necessário estudar as melhores condições para combinar as duas soluções em electrospinning co-axial. Conseguiu-se preparar fibras co-axiais que se confirmou terem um domínio exterior rico em silício e um interior rico em fósforo e sódio. Fizeram-se ensaios de produção de fibras co-axiais com enzima imobilizada, mas não se obteve evidência de atividade catalítica. Uma razão provável é a quantidade reduzida de membrana produzida, o que se traduz numa quantidade demasiado baixa de enzima. Foi difícil controlar a temperatura e humidade na câmara de electrospinning, mas melhorando o controlo destas variáveis, acredita-se que as formulações desenvolvidas poderão cumprir o objetivo de produção de uma membrana de fibras biocatalíticas co-axiais com HRP imobilizada para biorremediação.

Palavras chave: Biorremediação, co-axial, electrospinning, fibras, gelatina, líquido iónico, membranas biocatalíticas, oxido-redutase, sílica.

Nomenclature

[BMIM][BF_4]	1-butyl-3-methylimidazolium tetrafluoroborate
4-AAP	4-aminoantipyrine
Choline DHP	Choline dihydrogen phosphate
FTIR	Fourier transform infrared spectroscopy
HRP	Horseradish peroxidase
ILs	Ionic liquids
PSA	Sodium 4-hydroxybenzenesulfonate diehydrate
PVA	Poly(vinyl alcohol)
SEM	Scanning electron microscopy
TEOS	Tetraethyl orthosilicate
TMOS	Tetramethyl orthosilicate

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Chapter 1

State of Art

The increase in the production and use of chemicals that have occurred in recent decades has led to an increase in the number and amount of pollutants in the environmental.

1.1 Phenols as pollutants

Phenol and phenolic compounds (phenols) are produced in large amounts, due to their wide applicability in many industries [1]. Most of the phenols produced are used as intermediates to obtain a range of other compounds. Artificial resins consume most of the phenols produced, and are widely used in the construction, the automotive and appliance industries. Other chemical compounds manufactured from phenols find application in the synthesis of many other chemicals of interest to the medical, agro and textile industries, such as pharmaceuticals, disinfectants, fertilizers, pesticides, fibers, dyes. [2]–[5]

As a result of their widespread industrial use, phenols are common constituents of wastewaters that can easily infiltrate ecosystems. [6] Phenols discharged directly or indirectly into the environment may cause serious health problems. [7] Exposure to these substances is suspected to induce mutagenicity, teratogenicity, carcinogenicity, immunosuppression, endocrine disruption and infertility. [8], [9] Penetration of phenol into organisms is closely linked with diffusion of the compound across the cell membrane, facilitated by its hydrophobicity. In humans, phenol may irritate skin and cause its necrosis, damage kidneys, liver, muscle and eyes. Research on animal fibroblasts revealed mutagenic activity of phenol and bisphenol A. [10], [11] This compound also inhibited synthesis and replication of DNA in cells. [12] Catechol (a precursor to obtain pesticides, flavors, and fragrances) and hydroquinone (a developer for photography) were shown to inhibit DNA synthesis and reparation and thus stop activation and proliferation of T lymphocytes. [10] Medical data has shown that people exposed to chlorophenols developed tumors, sarcoma and lung cancer. [6] The mixture of chlorophenols or sodium salts of these compounds is probably carcinogenic for animals. [6] Phenols have also been shown to inhibit the growth or exert

lethal effects on aquatic organisms even at relatively low concentrations, depending on the temperature and state of maturity of the organism. [13]

Due to their toxicity, several phenols were taken into account by the US Environmental Protection Agency (US EPA) when it set wastewater standards for industry, as well as standards for all contaminants in surface waters, as part of pollution control programs. [14] Under The Clean Water Act (CWA; the EPA created the Priority Pollutant List. [2], [6] Phenol was one of the first entries to this list, which now includes 129 compounds. [6]

The most prevalent phenols in the environment are chlorophenols, formed by chlorination of mono and polyaromatic compounds present in soil and water, whose effects have been referred earlier. [6] The structures of some common phenolics are shown in Figure 1.1. Those that appear in the Priority Pollutant List are identified with a number indicating the order in which they appear in that list.

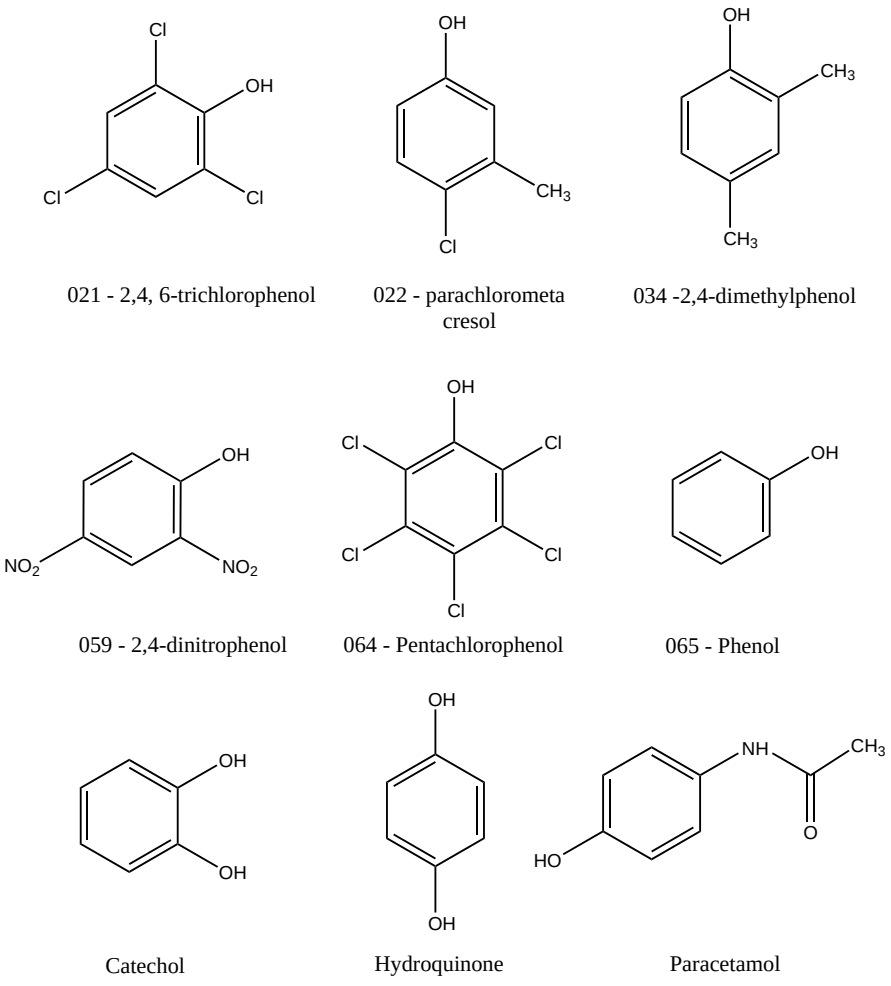


Figure 1.1: Chemical structures of phenols. Those with a number are included in the EPA Priority Pollutant List.

Due to the fact that phenols are aromatic compounds, their presence even at concentrations around 1 mg/L, can give rise to taste and odor problems in drinking water and food processing wastewater, making it unfit for use. [13], [15]

It should be noted that there are many phenolic compounds with recognized positive health effects, such as resveratrol, found in red grape skins, known for its activity as antioxidants. [16] The toxicity of phenols is associated with both their structure and concentration. [15]

1.2 Green Chemistry

There is widespread conviction that it is essential to decrease the environmental footprint of petroleum-based chemical processes, and concern over the effect of pollutants on the environment. Nowadays, leading scientists have many concerns about the long-term health risks associated with climate changes. The 2015 United Nations Climate Change Conference focused on measures to reduce greenhouse gas emissions, largely due to the release of carbon dioxide to the atmosphere. But many other topics in addition to air have been the object of regulations regarding pollution monitoring and control, such as water and land resources, as well as more specific topics such as waste, oil spills, pesticides, and other toxic substances (EPA). One important principle is that prevention is preferable to remediation. This is in fact one of the principles of "Green Chemistry", expressed in 1991 by P. T. Anastas in a special program launched by the EPA, aiming at implementing requirements for sustainable development in chemistry and chemical technology by industry. [17] In compliance with the notion of the importance of prevention, procedures that lead to the decrease, or eliminate the creation of pollutants or waste associated to materials or processes, are encompassed in the concept of green chemistry, or sustainable chemistry. [18]

The principles of green chemistry are listed in Table 1.1. The term "green chemistry" also refers to practices that reduce the use of hazardous and nonhazardous materials, energy, water and other resources, as well as protect natural resources through efficient use. [19]

In order to increase the sustainability of product industrial development, another new concept was created: ecodesign. This concept is based on different criteria such as economic, environmental, social and ethical aspects of the developed product, and was conceived to reach out to the 12 principles of green chemistry. *"Life cycle engineering (LCE) can be defined as the application of technological and scientific principles to the design and manufacture of products, with the goal of protecting the environment and resources, while encouraging economic progress, keeping in mind the need for sustainability, and at the same time optimizing the product life cycle and minimizing pollution and waste".* [21] The im-

Table 1.1: The 12 principles of Green Chemistry [20]

Number	Principle
1	Prevention
2	Atom Economy
3	Less Hazardous Chemical Syntheses
4	Designing Safer Chemicals
5	Safer Solvents and Auxiliaries
6	Design for Energy Efficiency
7	Use of Renewable Feedstocks
8	Reduce Derivatives
9	Catalysis
10	Design for Degradation
11	Real-time Analysis for Pollution Prevention
12	Inherently Safer Chemistry for Accident Prevention

plementation of green chemistry principles should lead to sustainable development locally and globally. Green chemistry is designed to actively contribute to best practices, and each person involved must contribute in order to build a future with sustainable natural resources. [18], [21]

1.3 Remediation of phenols

1.3.1 Conventional separation methods

The most widely used methods for removing phenols from wastewaters are activated carbon adsorption, coagulation–flocculation, ion-exchange, oxidation and electrochemistry. More recently, membrane-based processes, such as pervaporation, have also been shown to allow the removal of a considerable fraction of the organic pollutants. [5], [22] Treatments that use conventional separation methods are expensive and need a continuous input of chemicals. This makes them economically unfavorable, and causes further environmental damage. The search for news approaches to effluent/wastewater treatment has led to the development of bio-based processes.

1.3.2 Microbial bioremediation

Bioremediation is the process whereby organic wastes are biologically degraded under controlled conditions to levels below concentration limits established by regulatory authorities. [23] One definition for bioremediation is *"the use of living organisms, primarily microorganisms, to degrade the environmental contaminants into less toxic forms. It uses naturally occurring bacteria and fungi or plants to degrade or detoxify substances hazardous to human*

health and/or the environment". [24]

Environmental biotechnology is not a recent area of knowledge. Techniques such as soil treatment by composting, or wastewater treatment, are common examples of long used practices for reducing/eliminating hazardous substances. However, recent studies in molecular biology and ecology have led to more efficient biological processes. Remarkable achievements of these studies include the cleaning of polluted areas of water and land. [7] When microorganisms are used for bioremediation, many factors need to be taken into account for best results, such as temperature, pH, the presence of toxins, oxygen supply and available nutrients supply. The availability of nutrients may prevent microbes from targeting the contaminants of interest. There is also little mobility of the microorganism cells in the soil. And sometimes it is difficult to reach the contaminated site. The use of microbes for bioremediation may thus be compromised by many limitations. [25] Furthermore, costly and time consuming methods may be necessary to produce microbial cultures. [26] Oil spills are a good example to illustrate some of the difficulties encountered with microbial remediation. Oil spills are a great concern due to the amounts of oil released and their severe ecological impact. Microbial bioremediation using naturally occurring microbes in combination with mechanical approaches were used for removing polycyclic aromatic hydrocarbons (PAHs) from the Prestige ship oil spill on the north coast of Spain, in 2002. It is thought that, under suitable conditions, 50% or more of the oil from an oil spill may be degraded by microbes. [27] Unsuitable conditions, and as already referred, may lie in the difficulty to access the contaminated area, as in the case of Deepwater Horizon oil spill in 2010, and low temperature, which impaired microbial action towards the oil spill of the Exxon Valdez ship in Alaska, in 1989. When necessary, microorganisms may be imported to a contaminated site to enhance degradation, in a process known as bio augmentation. [23] But quite often naturally occurring microbes behave better than those fabricated in the lab. [27] Still, promise lies in the fact that there is still a lot of biodiversity to explore, and there must be many more useful microorganisms for bioremediation, whose functionalities are yet to be discovered.

1.3.3 Enzymatic bioremediation. Horseradish peroxidase

Microbial bioremediation relies on the action of microbial enzymes. There is evidence that many pollutants, from pesticides to dyes and drugs, can be degraded by isolated enzymes. [23] From an environmental standpoint, the use of enzymes instead of microorganisms can be advantageous:

I. Enzymes are simpler systems than microorganisms;

II. Enzyme catalyzed processes create less side products/waste;

III. Recent biotechnological advances have allowed the production of cheaper enzymes, on a larger scale, through better isolation and purification procedures;

IV. Using recombinant-DNA technology it is possible to obtain enzymes with enhanced stability, as well as enhanced activity or selectivity towards the target compounds, with a favorable cost-benefit ratio.

The search for enzymes that can target specific hazardous compounds usually involves looking for microbes capable of degrading those compounds, obtaining enzymatic extracts from those microbes, screening for the enzymatic activities of interest, and eventually separating and purifying the enzymes of interest. The development of enzyme processes for the treatment of wastewater has been the object of much attention [Durán2000], using in particular enzymes belonging to the EC (Enzyme Commission) 1 class, and called oxidoreductases. Oxidoreductases include oxygenases, monooxygenases, dioxygenases, laccases and peroxidases. [23] Horseradish peroxidase (HRP; Figure 1.2) can catalyze the oxidation of phenols, biphenols, anilines, benzidines and related hetereoaromatic compounds. [28] It is a promising candidate for the removal of phenols from industrial wastewaters due to its stability, broad substrate specificity, and its ability to operate at wide ranges of pH and temperature. [29]–[31]

Figure 1.2: Structure of horseradish peroxidase (1HCH from Protein Data Bank)

The use of peroxidases to remove phenolic compounds from aqueous solutions was firstly proposed by Klibanov and co-workers. [32] Since then, the method has been continuously improved.

The mechanism of peroxidase-catalyzed reactions starts with the binding of hydrogen peroxide to the iron atom, with formation of water and an enzyme intermediate. The latter

reacts with the reducing substrate (e.g. phenol), to form a second enzyme intermediate that similarly reacts with the reducing substrate with regeneration of the native enzyme. The process involves electron and proton transfer, and generates radical species that combine in a fourth step to give the product. [33]

1.4 Industrial applications of enzymes. Immobilization

The world's leading producer of industrial enzymes is Novozymes [34], with an estimated 48% share of the global enzyme market. The major uses of enzymes can be inferred by the volume of sales of Novozymes for each segment of applications, which are topped by household care formulations (laundry and dishwashing), followed by formulations for the food and beverages, bioenergy, agriculture and animal feed, and technical and pharma sectors.

The need to recover enzymes for reutilization, and to facilitate product purification are major reasons for enzyme immobilization. Another is to improve enzyme performance through changes in activity, selectivity, or stability. Stability is particularly important if the enzyme is expensive. Through immobilization it is possible to tune the enzyme microenvironment, and obtain a more adequate catalyst for the target application. [35], [36]

There is no universal immobilization method. The choice of the most appropriate immobilization technique for an enzyme depends on the enzyme itself, and on the objectives that must be achieved in terms of performance. The interactions established during enzyme immobilization may lead to a poor orientation of enzyme molecules, inducing a partial or a total loss of activity, e.g. through blocking of access to the enzyme active site. [35] Common immobilization methods are described in Table 1.2, and shown schematically in Figure 1.3.

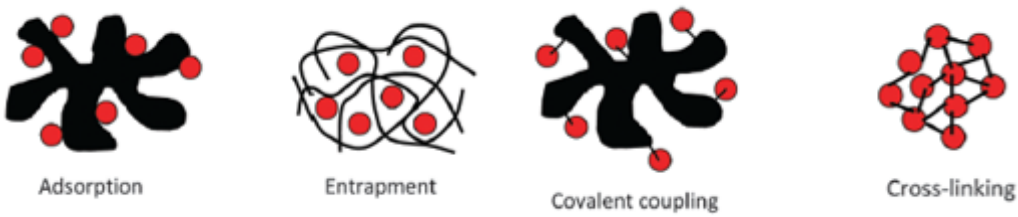


Figure 1.3: Common methods of enzyme immobilization [37]

Table 1.2: Advantages and drawbacks of main types of immobilization methods [35]

	Binding Nature	Advantages	Drawbacks	Examples
Adsorption	Weak bonds	Simple and easy; Limited loss of enzyme activity	Desorption; Non-specific adsorption	Physical adsorption; Electrostatic interactions; Layer-by-layer deposition; Electrochemical doping; Pre-immobilization on ion-exchange beads; Retention in a lipidic; Microenvironment
Covalent Binding	Chemical binding between functional groups of the enzyme and those on the support	No diffusion barrier; Stable; Short response time	Matrix not regenerable; Coupling with toxic product; High enzyme activity loss.	Activation of carboxylic groups; Activation of amino groups; Chemisorption
Entrapment	Incorporation of the enzyme within a gel or a polymer	No chemical reaction between the monomer and the enzyme that could affect the activity; Several types of enzymes can be immobilized within the same polymer	Diffusion barrier; Enzyme leakage; High concentrations of monomer and enzyme needed for electropolymerization	Electropolymerization; Amphiphilic network; Photopolymerization; Sol-gel; Polysaccharide-based gel; Carbon paste; Clay-modified electrodes
Cross-linking	Bond between enzyme/cross-linker (e.g. glutaraldehyde)/inert molecule (e.g. BSA)	Simple	High enzyme activity loss	

1.4.1 Adsorption

The easiest method for enzyme immobilization is adsorption. This technique is a physical immobilization onto a solid support. The enzyme is dissolved in a solution and the solid support is placed in contact with this solution for a given period of time. After this, the unadsorbed enzyme is removed by washing with buffer. The adsorption mechanisms are based on weak bonds, such as van der Waals forces and electrostatic and/or hydrophobic interactions. This technique does not involve any functionalization of the support and has generally little effect on enzyme activity relative to the free enzyme.

1.4.2 Covalent immobilization

Covalent binding of enzymes to polymeric supports is a very common immobilization method when enzyme stability is the target property to improve. It is widely used to

develop enzymatic biosensors. The biocatalyst is bound to the surface of the support through functional groups that are not essential for its catalytic activity.

1.4.3 Entrapment

This immobilization is easy to perform. Enzyme, mediators that form the structure within which the enzyme is entrapped, and additives can be placed in contact at the same time. The enzyme is in a medium that is adequate for its function when it is mixed with the other components, and therefore its activity is preserved during the immobilization process. Diffusion barriers can be a problem, with mass transfer limiting the rate of the overall reaction.

1.4.4 Cross-Linking

Immobilization of enzymes by cross-linking with bifunctional agents, such as glutaraldehyde, can lead to preparations said to be without support, since the enzyme is not immobilized with a comparatively larger mass of a different material, as is the case with the other immobilization methods The enzyme molecules can either be cross-linked with each other only, or with a functionally inert protein as well, such as bovine serum albumin. This method is easy to apply and the binding between enzyme molecules is strong. However, there may be a loss of enzyme activity due to the distortion of the active enzyme conformation.

1.5 Sol-gel

1.5.1 The process

The sol-gel process dates back to the 19th century, when an alkoxide was prepared from $SiCl_4$ and it started to form a gel when exposed to air. Later, this was found to be driven by atmospheric moisture, in a process involving the hydrolysis of the silicon alkoxide, followed by condensation. Over the years this process has been widely studied and it can be carefully tuned through manipulation of experimental conditions, such as the choice of catalyst (acid or base), precursor chemistries, number of precursors, the presence of additives, etc., coupled with strict control of physical and chemical phenomena involved in every step of the process.

The sol-gel process involves obtaining a three-dimensional network of an inorganic oxide

by chemical reaction. This network forms at relatively low temperatures. Starting from appropriate precursors, a colloid is formed - *the sol* - which can generate, by inorganic polymerization reactions, a three-dimensional rigid structure - *the gel* - from which a glassy or ceramic material can be obtained.

This process can lead to a wide variety of materials, from ultra-dense porous materials, to monoliths, powders, coatings, films and fibers, as shown in Figure 1.4. In the sol-gel process, there are many different ways that a gel can be formed. Sometimes, the same precursors can result in very different structures with only small changes in conditions. The tunability of microenvironment conditions that the sol-gel process allows is behind its widespread use in many fields of materials chemistry. [38]

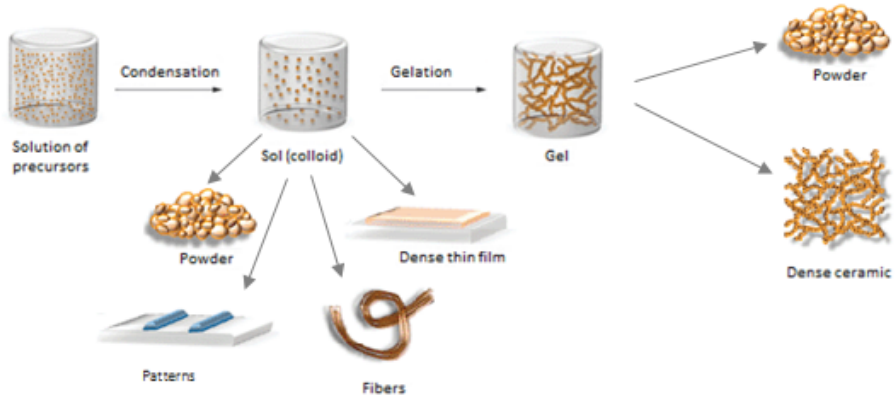


Figure 1.4: Representation of different chemical structures that can be obtained through the sol-gel process [38]

In more detail, formation of a sol-gel occurs through the following steps [38]:

I. Synthesis of the "sol" through hydrolysis and partial condensation of alkoxides;

II. Formation of the gel via polycondensation, with formation of metal-oxo-metal or metal-hydroxy-metal bonds;

III. "Aging" of the sol within the gel network, resulting in the elimination of the solvent;

IV. The drying of the gel causes the collapse of the porous network, forming a dense "xerogel"; the drying may be mediated by supercritical carbon dioxide, which avoids capillary stresses and results in the formation of an extremely low density "aerogel".

The precursor begins to undergo partial hydrolysis by reaction with water in the presence of a mutual solvent and a catalyst, according to equation (1.1)[39]

$$(RO)_3Si - OR + H_2O \underset{esterification}{\overset{Hydrolysis}{\rightleftharpoons}} (RO)_3Si - OH + ROH \tag{1.1}$$

After the formation of the first silanol groups (Si-OH), the process of condensation starts, with release of water or alcohol, leading to the formation of siloxane bonds, according to equations (1.2) and (1.3)[39]:

$$(RO)_3Si - OH + RO - Si(OR)_3 \underset{alcoholysis}{\overset{condensation}{\rightleftharpoons}} (RO)_3Si - O - Si(OR)_3 + ROH \tag{1.2}$$

$$(RO)_3Si - OH + HO - Si(OR)_3 \underset{Hydrolysis}{\overset{condensation}{\rightleftharpoons}} (RO)_3Si - O - Si(OR)_3 + H_2O \tag{1.3}$$

As these three reactions proceed, the dimensions of the colloidal oligomers increase, and by coming into contact with one another, they form agglomerates. The gel point corresponds to the appearance of a single cluster, expanding, coexisting with the colloidal phase (the sol), and still containing many clusters of smaller dimensions. [39] Through polycondensation, a three dimensional network of silica is formed, which can immobilize species in its interstices. When the sol-gel is obtained using an organic solvent, such as an alcohol, the resulting material is called an "alcogel". [39]

One of the problems of sol-gel entrapment can be restrained access to the enzyme that is confined within the solid matrix. Therefore the porosity of the material should be carefully controlled, through the choice of precursors and additives. As already mentioned, an important parameter in the synthesis of sol-gel materials is the catalyst, namely acidic or basic, which affects the structural rearrangement that can be obtained in the process, as shown schematically in Figure 1.5.

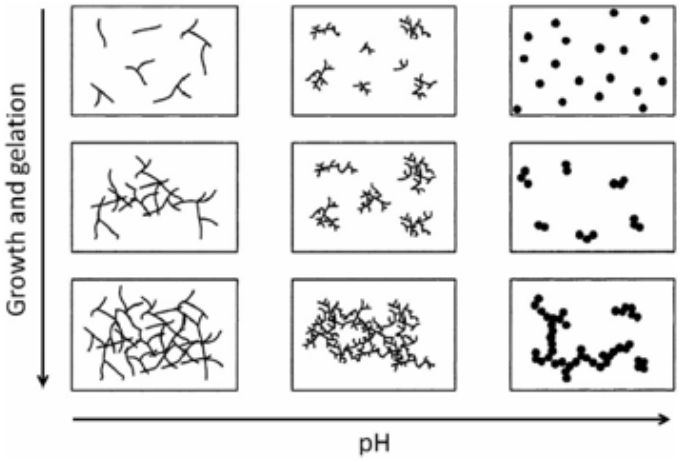


Figure 1.5: Effect of pH and aging time on sol-gel structure [40]

In this work, attention will be directed only to sol-gel materials obtained through acid catalysis, as is shown schematically in Figure 1.6.

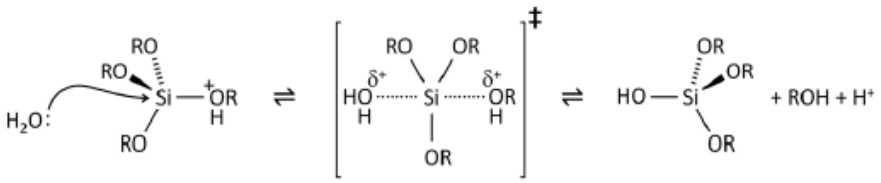


Figure 1.6: General mechanism of hydrolysis and condensation of alkoxysilane precursors to form silica in acid catalyzed conditions [41]

1.5.2 Bio-applications of sol-gel materials

As early as 1955, Dickey conducted studies on the immobilization of enzymes within sol-gel matrices. [42] But it took nearly 40 years for the scientific community to realize the potential of this immobilization technique for biological materials, as highlighted by Braun et al. [43], in an article describing the properties of entrapped enzymes in TEOS-derived sol-gel matrices.

For enzymes to be useful in industrial processes, they must be stable enough to withstand reutilization. [44] Using the sol-gel process, it is possible to obtain catalysts with good performance, including high resistance to thermal denaturation, significant improvement in enzymatic activity, and log-term stability. [45] Confinement within a sol-gel matrix is particularly suited to the immobilization of multi-enzymatic systems, in which the product of one enzymatic reaction is the substrate for the next enzymatic reaction in the sequence. [46]

One remarkable aspect of the sol-gel process is that it is well suited for the entrapment of very sensitive species. Indeed, many different biological molecules and systems, including DNA, RNA, antibodies, and viable, living cells, from bacteria, yeast, plants, have been encapsulated in glasses of silica or other metallic oxides, organosiloxanes, and many other sol-gel hybrids or composite polymers. [44], [47]–[50] In these cases, the experimental conditions of the sol-gel process must be carefully optimized to preserve the activity, or function, of the immobilized biomolecule or system. [51], [52]

1.6 Ionic liquids

The first industrial process involving an ionic liquid (IL) was announced in March 2003, and since then recognition of the potential of ILs for basing new chemical processes increased dramatically. ILs are probably one of the most studied chemical compounds in the last decade. [53]

ILs are composed entirely of ions. To distinguish them from molten salts, which is a designation used for common salts in liquid form, often at very high temperatures, the designation "room temperature ionic liquids" (RTILs) is sometimes used, given that ILs have melting points around or below 100 °C. [53]

One of the most highly valued properties of many ILs, and probably their "greenest" property, is their extremely low or immeasurable vapor pressure. [54] This contrasts with many of the solvents still used by industry, which are volatile organic compounds (VOCs). Replacement of conventional solvents by ILs would prevent the emission of VOCs, which are a big source of environmental pollution. [54] But ILs have many other interesting properties. An infinite number of ILs can in principle be prepared, by combining a cation with an anion. This tunability allows the synthesis of ILs with different conductivity, polarity, miscibility behavior, and hydrophobicity/hydrophilicity. [55]

Many ILs have been shown to support enzyme activity. However, when the ions of the IL coordinate strongly with the enzyme, the IL normally has a negative effect on enzyme activity. Many ILs can dissolve greater amounts of water than many organic solvents, and can thus strip water from an enzyme. It is important to ensure that the level of hydration of the enzyme is adequate for its function.

There are also reports on increased stability of biological materials in ILs. [56] For example, phosphate (choline DHP) has been shown to support the retention of structure and activity of proteins such as cytochrome c, lysozyme, and ribonuclease A. [57], [58] Weaver et al. used water/choline DHP mixtures in which lysozyme and interleukin-2 were soluble, and

saw that these proteins were more stable than in aqueous solutions. The presence of a sufficient amount of water in these systems is pointed out as an important factor as regards protein solubility and stability. [58]–[62]

The first reports on the use of enzymes in ILs were focused on hydrolases, such as proteases and lipases, which are the type of enzymes most used in industrial applications. Later reports included oxidoreductases, such as peroxidases and dehydrogenases. One important evidence of some of the first studies conducted was that enzymes that worked in organic solvents also worked in ILs. [53]

1.6.1 Ion jelly

The Ion jelly is a material that combines the gelling properties of gelatin with the chemical properties of ILs. The gelatin and the IL establish stable hydrogen bond interactions upon crosslinking, which allows the retention of water. This approach was developed by Vidinha et al. and allows to obtain materials which can be applied in various fields. [63] Gelatin is an inexpensive and well-studied gelling agent. ILs, on the other hand, are "designer solvents" that can be synthesized with target properties according to the need, through the choice of anion and cation.

Due to the presence of the IL, Ion jelly materials are conductive, and are thus suitable for application in electrochemical devices. They are also adequate for the immobilization of enzymes whose mechanism involves proton and electron exchange, as is the case with oxidoreductases. [53], [55]

1.7 Electrospinning

What we now call electrospinning is a rather old technique developed and patented by Cooley and Morton in 1902. Advances in materials science over the last decades, mainly since the late 1990s, have drawn attention to the huge potential of the process for nanofiber production (Doshi and Reneker 1995). [64] In fact, electrospinning is a simple and versatile method to produce fibers, using several materials, such as polymers, composites and ceramics. This flexibility has made possible the application of fibers and membranes in many different fields, such as traditional filtration, the manufacturing of biosensors, protective clothing, energy conversion systems, cosmetics, drug delivery systems, medical, electronic and optical devices, tissue engineering, food items, etc. [65]–[67] Some of these applications are highlighted in Figure 1.7. [68]

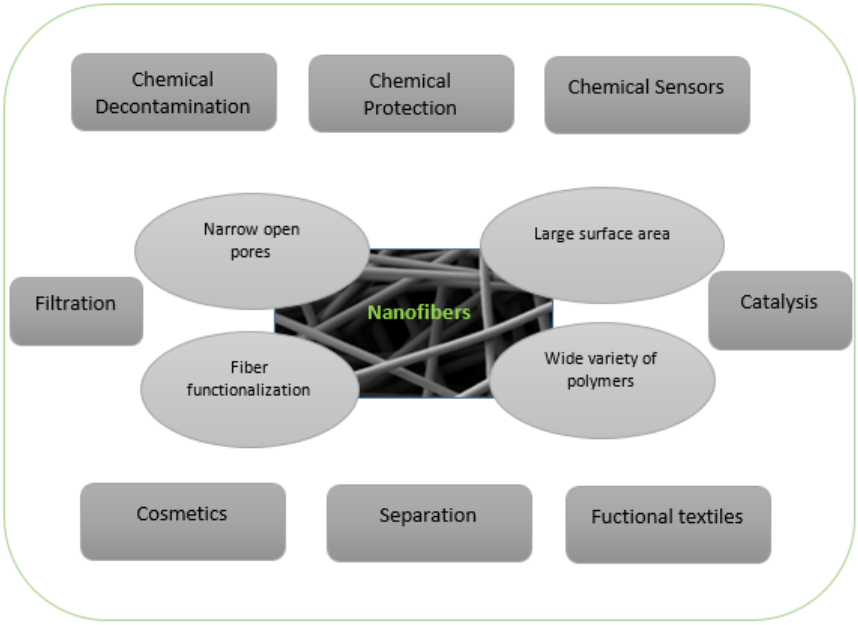


Figure 1.7: Representation of advantages and applications of fibers [67]

Some of the advantages of electrospun fibers are indicated below:

I. **High surface area to volume ratio**
The nano-dimension of a nanofiber gives it a high surface area to volume ratio. This ability supports applications where a large surface area is desirable. [69]

II. **Wide variety of polymers and materials**
Electrospinning has been used to make nanofibers from all classes of materials. Although the process is predominantly used to make polymeric nanofibers, ceramic and metal nanofibers have also been constructed. [70], [71]

III. **Easiness of fiber functionalization**
To obtain nanofibers with a given functionalization, the functionalization agent may be mixed in the electrospinning solution, or applied post-spinning to the surface of the membrane produced. Another approach is using core-shell electrospinning setup. [72]

IV. **Variety of combination of materials**
Different materials can be easily mixed together for spinning into fibers. [46]

V. **Easy fiber deposition onto other substrates**
Deposition of electrospun fibers requires the collecting surface to have a lower static charge. Electrospun fibers have been routinely deposited on surfaces such as metal, glass, microfibrous mats and water.

VI. **Straightforward fabrication of nanofibrous structures and different morphologies**
Advances in electrospinning setup and process allow to obtain tubular nanofibrous structures, yarns and 3D blocks of nanofibers. The electric field influences the morphology of the fibers obtained. [73]–[75]

VII. **Demonstrated mass production capability**
Several companies have used electrospinning to spin nanofibrous membranes at an industrial level. Electrospinning setups for mass production of nanofibers are also commercially available. [76]

Through the blending of different components in the electrospinning solution, and through changes in experimental electrospinning conditions, such as electric potential, humidity of the chamber, it is possible to make fibers with different composition, morphologies, strength, diameters, porosities, conductivity. The optimization of the electrospinning polymer solution is crucial, as regards viscosity, conductivity, and aging conditions. [40], [77], [78]

Using electrospinning, biocatalytic membranes with immobilized enzymes have been manufactured, for applications in biosensors and bioreactors. [35], [79] The porous membranes, working as a selective barrier as well as a support for enzyme immobilization, allowed enzyme reuse, led to increased enzyme stability, and allowed continuous processing. [80] High surface area and small pore size were important features of electrospun, nanofibrous, biocatalytic membranes prepared for the removal of different contaminants from wastewaters. [81]

Silica fibers have several advantages over other materials due to the fact that silica is nontoxic and highly compatible with a range of biological species such as enzymes, proteins, peptides and drugs. [82][83] In addition, silica is chemically inert, and is thermally and mechanically stable. [84]

1.7.1 The electrospinning technique

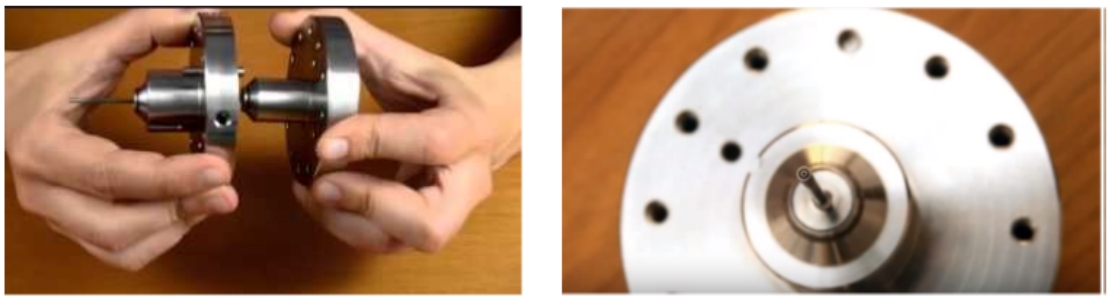
When a high voltage is applied to the droplet solution, the molecules of this droplet become charged and an electrostatic repulsion occurs, which counteracts the surface tension of the droplet. When the high voltage crosses a critical point, a jet of the solution is disrupted from the surface. The droplet that emerges from the end of the needle is deformed into a conical shape, normally called the "Taylor cone". The elongation and thinning of the charged jet due to instability leads to the formation of continuous fibers with diameters at

the nanoscale.

Based on this principle, different electrospinning setups as well as different types of collectors have been designed to create various nanofibrous architectures. Parameters such as electric potential, flow rate, distance between the needle and collection surface, ambient parameters (temperature, humidity and air velocity/extraction in the chamber) must be taken into account. For instance, the polymer solution must have a concentration high enough to cause polymer entanglements induced by the electric field. Thus there is an optimum voltage for stable spinning without any surface perturbations in the conical base region of the solution. [66], [85] At a laboratory level, a typical electrospinning setup only requires a high voltage power supply (range between1 and 30 kV), a syringe, a needle, a conducting collector, and a chamber that prevents changes in parameters, such as temperature and humidity.

1.7.2 Co-electrospinning for making core-shell fibers

Co-electrospinning allows the manufacturing of core-shell nanofibers using multiple needles, as shown in Figures 1.8a and 1.8b. Bioactive agents have already been encapsulated in the cores of nanofibers, such as drugs, proteins and enzymes. [86] Core-shell fibers provide two different environments. The core of the fiber can be adapted to the requirements of the target biological product, while the shell provides protection and extra stability. High surface area and adequate porosity, allowing the transfer of solutes but not of biological molecules, make this approach particularly suited for enzymes. One such example is the work of Tong and co-workers who encapsulated cells in core-shell fibers with a silica shell. [81] The experimental setup for doing co-electrospinning is similar to the setup used for electrospinning, but uses different needles. [87]–[89]



(a) Coxial setup (b) Needles

Figure 1.8: Coxial apparatus

1.8 Goals of this dissertation

The aim of the present work was to manufacture a core-shell electrospun fiber membrane, with a silica shell obtained through the sol-gel process, and a core providing an adequate microenvironment for immobilizing an enzyme.

It was necessary to optimize the electrospinning shell solution as regards the silica precursors (TMOS and TEOS), and the silica/water/methanol/HCl/PVA ratio. The presence of PVA was to give the fibers flexibility and porosity, while keeping the strength imparted by silica. It was also necessary to optimize the electrospinning core solution. It was intended to make fibers that could be used in both nonaqueous and aqueous media, and therefore it was important that fibers were insoluble in water. With that objective, Ion jelly was chosen to make the core of the fibers. The enzyme to be immobilized was an oxidoreductase, which, as was already mentioned, transfers protons and electrons, and a charged microenvironment should be beneficial. Also the Ion jelly, depending on its composition, is not water soluble. The oxidoreductase selected was horseradish peroxidase, an enzyme that degrades phenols, as referred earlier. The goal was to manufacture a core-shell fiber membrane that could be used for bioremediation.

Chapter 2

Materials and Methods

2.1 Chemicals

TMOS (tetramethyl orthosilicate; $Si(OCH_3)_4$; wt = 152.25 g/mol; density = 1.032 g/cm^3; $\geq$ 98%), TEOS (tetraethyl orthosilicate; $Si(OC_2H_5)_4$; wt = 208.33 g/mol; density = 0.933 g/cm^3; $\geq$ 99%), methanol (wt = 32.04 g/mol; density = 0.792g/cm^3; 99.8%), ethanol (wt = 46.07 g/mol; density = 0.789 g/cm^3), polyvinyl alcohol (PVA; 87-90% hydrolyzed, wt = 30000-70000 g/mol, taken to be 50000 g/mol in all calculations), sodium acetate trihydrate (wt = 136.08 g/mol; $\geq$ 99%), sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$; wt = 137.99 g/mol; $\geq$ 98%), 4-aminoantipyrine (4-AAP; $C_{11}H_{13}N_3O$; wt = 203.24 g/mol; $\geq$ 99%), citric acid (wt=192.12 g/mol; $\geq$ 99.5%) were from Sigma-Aldrich, hydrochloric acid (HCl; wt = 36.46 g/mol; density = 1.19 g/cm^3; $\geq$ 38%) and di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$; wt = 177.99 g/mol; $\geq$ 99%) were from Scharlau, acetic acid, glacial (wt = 60.05 g/mol; density = 1.049 g/cm^3; 99.8%) was from Carlo Erba, gelatin from porcine skin (90-110 Bloom) was from Oxoid, the ionic liquids (ILs) choline dihydrogen phosphate (choline DHP; 98%) and 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF_4]) were from IoLiTec, sodium 4-hydroxybenzenesulfonate dihydrate (PSA; $C_6H_5NaO_4S \cdot 2H_2O$; wt = 232.20 g/mol; $\geq$ 97%) was from Alfa Aesar, potassium hydroxide lentils (wt = 39.997 g/mol; $\geq$ 85%) was from Bio Express, sodium hydroxide (wt=40.00g/mol $\geq$ 98%) was from AkzoNobel Eka, hydrogen peroxide (H_2O_2; wt = 34.01 g/mol; density = 1.11 g/cm^3; 30% w/v) was from Panreac AppliChem. Ammonia (wt = 17.03g/mol; density = 0.91 g/cm^3; 25% w/v) from Pronalab. The water used was Millipore water. The enzyme was horseradish peroxidase (HRP; P8125; bottle with 25 000 units, where 1 pyrogallol unit will form 1.0 mg purpurogallin from pyrogallol in 20 s at pH 6.0 at 20 °C), from Sigma-Aldrich

2.2 Development of sol-gel solutions for testing in electrospinning

When used, the term sol-gel solution always refers to the blend of the silica precursor TMOS or TEOS with water and acid (mostly, HCl). The sol-gel solution usually contains PVA, and may also contain methanol or ethanol (for TMOS or TEOS, respectively), as well as other additives, such as acids. The mixing of the components of the mixture is usually achieved using magnetic stirring at moderate speeds (65-100 rpm).

The preparation of sol-gel solutions was made based on the choice of given molar ratios of the components, following and adapting different protocols.

2.2.1 TMOS-based sol-gel solutions without PVA

Based on the protocol described by Obert and Dave [90] a typical preparation consisted in mixing, in one step, 250 μL of TMOS with 306 μL of water and 3.5 μL of HCl solution (0.04 M), as indicated in Table 2.1.

Table 2.1: Molar ratios used for preparing sol-gel solutions without PVA

	TMOS	**H_2O**	**HCl**
a)	1.00	10.01 (1.82+8.19)	8.26×10^{-5}
b)	1.00	10.01	8.26×10^{-5}
c)	1.00	10.01	3.30×10^{-4}
d)	1.00	22.93	8.26×10^{-5}
e)	1.00	10.01 (1.82+8.19)	8.26×10^{-5}
f)	1.00	10.01	8.26×10^{-5}
g)	1.00	10.01	8.26×10^{-4}
h)	1.00	1.82	8.26×10^{-4}
i)	1.00	4.09	7.38×10^{-3}
j)	1.00	1.82	8.26×10^{-5}
k)	1.00	16.38	8.26×10^{-5}

Sol-gel solutions a) and e) were prepared by adding, to TMOS, 56 μL of H_2O and the HCl solution required, and sonicating for 20 minutes, after which 250 μL of water were added and well mixed with the tip. After this, the preparation was left at room temperature.

Sol-gel solution i) was prepared as in a typical case, but was left for 24 h at 4 °C after preparation.

Sol-gel solutions e), f) and k) were prepared as in a typical case, but after sonication the mixtures were stirred in a vortex for 40 s, and then put in an ice bath for 10 min. In j), the

difference was that after vortex shaking for 40 s, the mixture was left at room temperature.

2.2.2 TMOS-based sol-gel solutions with PVA

To study the influence of PVA, 10%, 15% and 18% (w/w) PVA solutions were prepared. For example, a 10% (w/w) PVA solution was obtained by adding 200 mg of PVA to 1800 μL of water. The mixtures were kept under magnetic stirring, at 60 °C, for 1 h.

A typical preparation consisted in mixing 250 μL of TMOS with 306 μL of water, and adding 3.5 μL of HCl solution (0.04 M) dropwise. The mixture was then heated, under magnetic stirring, at 60 °C, for 1 h. After this step, 72 μL of PVA (10%) solution kept at room temperature were added, and the mixture was well stirred for a few seconds. Then the stirring was stopped and the mixture was left at room temperature. Several volumes of PVA solution (amounts of PVA) were added, as indicated in Table 2.2.

Table 2.2: Molar ratios used for preparing sol-gel solutions with PVA - I

	TMOS	H_2O	HCl	PVA	Notes
l)	1.00	10.01	8.26×10^{-5}	8.54×10^{-5}	
m)	1.00	10.01	8.26×10^{-5}	7.64×10^{-2}	
n)	1.00	10.01	8.26×10^{-5}	7.64×10^{-2}	The sol-gel solution was not heated
o)	1.00	5.73	4.43×10^{-3}	7.64×10^{-5}	The sol-gel solution was heated at 58 °C for 35 min
p)	1.00	5.73	4.43×10^{-3}	2.70×10^{-4}	The sol-gel solution was heated at 55 °C for 1 h 15 min
q)	1.00	5.73	4.43×10^{-3}	4.57×10^{-5}	The sol-gel solution was heated at 55 °C for 1 h 15 min
r)	1.00	5.73	1.48×10^{-3}	-	The sol-gel solution was heated at 63 °C
s)	1.00	5.73	4.43×10^{-3}	-	The sol-gel solution was heated at 50 °C for 2 h
t)	1.00	5.73	2.95×10^{-3}	-	The sol-gel solution was heated at 62 °C
u)	1.00	5.73	4.43×10^{-3}	-	The sol-gel solution was heated at 58 °C

In cases r), t) and u), the sol-gel solution became solid before it completed 1 h at 60 °C, and the PVA solution was not added. In case s), no PVA was added, to measure the time it took for the sol-gel solution to become solid.

The temperature at which the sol-gel solution was prepared influenced its properties, namely the time it remained fluid. To study this effect, the previous protocol was changed, and new sol-gel solutions were tested, as indicated in Table 2.3.

Table 2.3: Molar ratios used for preparing sol-gel solutions with PVA - II

	TMOS	**H_2O**	**HCl**	**PVA**	**Temperature of mixing step (1 h)**
1.a)	1.00	5.73	1.48×10^{-3}	1.38×10^{-4}	50 °C
1.b)	1.00	5.73	1.48×10^{-3}	2.06×10^{-4}	50 °C
1.c)	1.00	5.73	1.48×10^{-3}	1.97×10^{-4}	50°C
1.d)	1.00	5.73	1.48×10^{-3}	2.94×10^{-4}	50 °C
2.a)	1.00	5.73	1.48×10^{-3}	1.38×10^{-4}	60°C
2.b)	1.00	5.73	1.48×10^{-3}	2.06×10^{-4}	60 °C
3.a)	1.00	5.73	4.43×10^{-3}	1.38×10^{-4}	50°C
3.b)	1.00	5.73	4.43×10^{-3}	2.06×10^{-4}	50 °C
3.c)	1.00	5.73	4.43×10^{-3}	1.97×10^{-4}	50°C
3.d)	1.00	5.73	4.43×10^{-3}	2.94×10^{-4}	50 °C
4.a)	1.00	4.09	7.38×10^{-4}	4.54×10^{-4}	50°C or room temperature
4.b)	1.00	4.09	7.38×10^{-4}	3.63×10^{-4}	50 °C or room temperature
5.a)	1.00	4.09	7.38×10^{-4}	6.81×10^{-4}	50°C or room temperature
5.b)	1.00	4.09	7.38×10^{-4}	5.45×10^{-4}	50 °C or room temperature
6	1.00	4.79	1.25×10^{-3}	-	60°C
7	1.00	5.73	4.43×10^{-3}	-	60 °C
8	1.00	4.79	1.25×10^{-3}	-	50 °C

For example, mixtures labelled 1 were prepared with 400 μL TMOS, 280 μL water and 40 μL HCl (0.10 M) added dropwise, in a water bath, at 50 °C, under magnetic stirring, for 1 h, after which the PVA (10%) solution was added, and the mixtures were left at room temperature. Typically, to 100 μL of the sol-gel solution were added x μL of the PVA solution (in the case of solution 1.a), x = 26 μL).

Mixtures 6, 7 and 8 were used for the same purpose as mixture s) in Table 2.3, to measure the time it took for the sol-gel solution to become solid. In particular, mixtures 7 and 8 were prepared with 200 μL TMOS, 100 μL water and 10 μL HCl (0.10 M). Each one of these mixtures was divided in two, one part kept in a water bath at the temperature referred in the table, and the other part kept at room temperature. Mixture 7 was kept for 1 h in the water bath, and mixture 8 for 2 h.

Through the knowledge obtained from previous experiments with sol-gel solutions, another protocol was tested. A typical procedure was to mix 1500 μL of TMOS and 1050 μL of water, and adding 150 μL of HCl (0.1 M) dropwise. After blending all the components, the mixture was heated at 60 °C for 1 h, under magnetic stirring. The PVA aqueous solution was mixed with the sol-gel solution right before putting the solution in the syringe. Conditions were as indicated in Table 2.4.

Table 2.4: Molar ratios used for preparing sol-gel solutions with PVA – III

	TMOS	H_2O	HCl	PVA	Notes
i)	1.00	5.73	1.48×10^{-3}	1.06×10^{-1}	-
ii)	1.00	5.73	1.48×10^{-3}	6.64×10^{-2}	-
iii)	1.00	5.73	1.48×10^{-3}	8.85×10^{-2} $(6.64 \times 10^{2} + 2.21 \times 10^{-2})$	2 additions, 20 min apart
iv)	1.00	5.73	1.48×10^{-3}	6.64×10^{-2}	Sol-gel solution prepared at 45 °C for 45 min
v)	1.00	5.73	1.48×10^{-3}	8.85×10^{-2} $(6.64 \times 10^{2} + 2.21 \times 10^{-2})$	Sol-gel solution prepared at 45 °C for 45 min
vi)	1.00	5.73	1.48×10^{-3}	15.92×10^{-5} $(7.96 \times 10^{-5} + 7.96 \times 10^{-5})$	Sol-gel solution prepared at 60 °C for 50 min. 2 additions, 20 min apart
vii)	1.00	5.83	1.50×10^{-3}	2.48×10^{-4}	-
viii)	1.00	5.83	1.50×10^{-3}	3.74×10^{-4}	-
ix)	1.00	5.83	1.50×10^{-3}	4.66×10^{-4}	-
x)	1.00	5.83	1.50×10^{-3}	5.63×10^{-4}	-

In cases vii) to x), the amount of solid PVA required for the molar ratio selected was added to the sol-gel solution, kept in a water bath at 60 °C, under magnetic stirring, for 30 min.

2.2.3 TMOS-based sol-gel solutions with PVA and methanol

Following the work of Pizarda [91], sol-gel solutions were prepared according to a procedure developed for TEOS-based formulations, which will be described in section 2.3. This procedure was changed based on the observation of the fibers obtained, namely as regards the amounts of the HCl and the PVA solutions used. The amount of HCl was decreased, and the amount of PVA was increased (Pizarda et al. [91] used a PVA with a molecular weight of 205000 g/mol, which is four times higher than the molecular weight of our PVA).

A typical sol-gel solution was thus prepared by mixing 1000 μL TMOS, 300 μL methanol, 245 μL water and 13.3 μL of HCl (4 M), in a water bath at 60 °C, for 50 min, under magnetic stirring. Then 650 mg of PVA (18%) solution were added, and the resulting mixture was kept in the water bath, under stirring, for 1 h 15 min. Assays were also performed without methanol. Conditions were as indicated in Table 2.5.

Table 2.5: Molar ratios used for preparing sol-gel solutions with PVA and methanol

	TMOS	H_2O	Methanol	HCl	PVA
1.	1.00	2.01	1.09	7.85×10^{-3}	3.45×10^{-4}
2.	1.00	5.74	1.09	4.13×10^{-2}	3.45×10^{-4}
3.	1.00	5.17	0.99	4.13×10^{-2}	3.45×10^{-4}
4.	1.00	2.80	-	4.33×10^{-4}	3.72×10^{-5}
5.	1.00	2.80	-	4.33×10^{-4}	1.05×10^{-4}

2.2.3.1 TMOS-based sol-gel solutions with PVA and methanol, with enzyme

To prepare silica fibers with entrapped enzyme, a typical preparation was the sol-gel solution 1 in Table 2.5. Just before putting this mixture in the electrospinning syringe, 100 μL of a 10 mg/mL solution of enzyme in phosphate buffer at pH 6.0 was added and mixed with the tip.

2.2.4 TMOS-based sol-gel solutions with PVA and acetic acid

Another approach for preparing sol-gel solutions for electrospinning was made, following [92]. In this case, a typical procedure was mixing 736 μL TMOS and 180 μL water, and adding 30 μL HCl (0.04M) dropwise, in a water bath at 60 °C, for 30 min, under magnetic stirring. The mixture was left to cool at room temperature, after which 900 μL of PVA (18%) solution were added, and the mixture was stirred again for around 2 min. To prevent fast solidification, 15 μL of a 100 mM solution of acetic acid were added together with the PVA solution. Conditions are indicated in Table 2.6.

Table 2.6: Molar ratios used for preparing sol-gel solutions with PVA and acetic acid

	TMOS	H_2O	HCL	Acetic acid	PVA
1.	1.00	2.00	2.41×10^{-4}	-	4.78×10^{-3}
2.	1.00	2.00	2.41×10^{-4}	-	3.72×10^{-3}
3.	1.00	2.00	2.41×10^{-4}	-	1.80×10^{-3}
4.	1.00	2.00	2.41×10^{-4}	3.01×10^{-4}	2.52×10^{-3}
5.	1.00	2.00	2.41×10^{-4}	6.02×10^{-4}	7.08×10^{-3}

2.2.5 TEOS-based sol-gel solutions with PVA, ethanol and citric acid

The preparation of sol-gel solutions using TEOS was made by adapting a protocol for the production of bioglass [93]. The basis for the various preparations was mixture I in Table 2.7. In this case, 1.33 mL of TEOS were mixed with 6.00 mL of ethanol and 9.00

mL of water, for 45 min under magnetic stirring, at room temperature. Then the citric acid solution was added to bring the pH to around 2 (approximately 35 mL of citric acid solution, prepared by dissolving 2.40 g of citric acid in water at room temperature, under stirring, and adjusting the final volume to 50.0 mL), and stirring was maintained for 1 h at room temperature.

Mixture IV was made by addition of 400 μL TEOS, 1800 μL ethanol, 2700 μL of water and 112 μL of HCl (0.1 M) instead of citric acid. After this, the solution kept being stirred for 45 min at room temperature.

Mixture v) was made similarly to mixture I), but the solution of citric acid was four times more concentrated (4.81 g citric acid in 25.0 mL of solution). The volume of this solution was 800 μL.

Table 2.7: Molar ratios used for preparing sol-gel solutions with PVA, ethanol and citric acid

	TEOS	H_2O	Ethanol	Citric acid	PVA	Notes
I))	1.00	410.08	17.26	51.07	-	
II))	1.00	410.08	17.26	51.07	-	After adding citric acid, the sol-gel solution was heated for 1 h at 60 °C (*)
III))	1.00	410.08	17.26	51.07	3.11×10^{-2}	
IV)	1.00	100.50	17.26	-	-	Prepared with HCl instead of citric acid
V)	1.00	91.33	17.26	204.28	-	
II)a.)	1.00	91.33	17.26	51.07	2.79×10^{-3}	(*)
II)b.)	1.00	91.33	17.26	51.07	4.48×10^{-3}	(*)
II)c.)	1.00	91.33	17.26	51.07	5.64×10^{-3}	(*)
II)d.)	1.00	91.33	17.26	51.07	8.31×10^{-3}	(*)
IV)a.)	1.00	84.92	17.26	-	2.79×10^{-3}	Prepared with HCl instead of citric acid
IV)b.)	1.00	84.92	17.26	-	4.48×10^{-3}	Prepared with HCl instead of citric acid
IV)c.)	1.00	84.92	17.26	-	5.64×10^{-3}	Prepared with HCl instead of citric acid
V)a.)	1.00	91.33	17.26	204.28	2.79×10^{-3}	
V)b.)	1.00	91.33	17.26	204.28	4.48×10^{-3}	
V)c.)	1.00	91.33	17.26	204.28	5.64×10^{-3}	
V)d.)	1.00	91.33	17.26	204.28	8.31×10^{-3}	

2.2.6 TEOS-based sol-gel solutions with PVA and ethanol

An illustrative preparation of a sol-gel solution was made following the work of Pizarda [91], as indicated in Table 2.8. It involved mixing 1000 μL TEOS, 236 μL ethanol, and 170 μL H_2O, and adding 35 μL HCl (4 M) dropwise. The resulting mixture was heated at 60 °C for 1 h, under stirring. To this mixture were added 659 mg of PVA, under stirring, to make the mixture 18% in PVA.

Table 2.8: Molar ratios used for preparing sol-gel solutions with PVA and ethanol

	TEOS	**H_2O**	**Ethanol**	**HCL**	**PVA**
1.	1.00	2.10	9.00×10^{-1}	1.40×10^{-4}	5.29×10^{-1}
2.	1.00	2.11	9.00×10^{-1}	2.80×10^{-4}	5.29×10^{-1}
3.	1.00	2.10	9.00×10^{-1}	3.31×10^{-7}	6.17×10^{-4}
4.	1.00	2.10	9.00×10^{-1}	3.31×10^{-7}	4.32×10^{-4}
5.	1.00	8.81	1.43	2.80×10^{-4}	1.40×10^{-4}
6.	1.00	9.64	1.16	2.80×10^{-4}	5.29×10^{-1}

2.3 Development of mixtures without silica for testing in electrospinning

2.3.1 Mixtures based on Ion jelly

A typical preparation for making fibers consisted in mixing 300 μL acetic acid, glacial, with 430 mg sodium acetate, 250 mg gelatin, 111 mg choline DHP, and 700 μL phosphate buffer at pH 6, in a water bath at 60 °C, and mixing with a magnet for 1 h.

Table 2.9: Quantities used for making gelatin fibers with IL

	Acetic acid, glacial	**Sodium acetate**	**Water**	**Gelatin**	**Phosphate buffer**	**Choline DHP**
1.	300 μL	430 mg	700 μL	250 mg	-	-
2.	300 μL	430 mg	-	250 mg	700 μL	-
3.	300 μL	430 mg	-	111 mg	700 μL	111 mg
4.	300 μL	430 mg	-	250 mg	700 μL	111 mg
5.	150 μL	215 mg	-	250 mg	850 μL	111 mg
6.	-	-	1000 μL	200 mg	-	250 mg

2.3.2 Mixtures based on Ion jelly, with enzyme

A typical procedure was to follow the protocol described in 2.3.1, allowing the mixture to cool down to 35 °C, and just before putting the mixture in the electrospinning syringe, 100 μL of a 10 mg/mL solution of enzyme in phosphate buffer at pH 6.0 were added and mixed with the tip.

2.3.3 Mixtures based on PVA

For trying out the PVA solutions for making fibers, two different concentrations were tested, namely 18% and 25%.

2.4 Mixtures for preparing silica-shell/Ion jelly-core fibers

To make co-axial fibers, sol-gel solutions and gelatin mixtures were prepared as those already tested in conventional electrospinning. A typical sol-gel solution was obtained by mixing 5.0 mL of TMOS, 1.50 mL methanol, 1.225 mL of water and 67 μL of HCl (4 M), in a water bath at 60 °C, under magnetic stirring, for 50 min. Then 3.25 g of PVA solution at 18% were added, and the resulting mixture was kept under attiring at 60 °C, for 1 h 30 min. Two different approaches were followed to prepare the gelatin mixture:

A) 333 mg of gelatin and 333 mg of IL (choline DHP) were mixed with 3.0 mL of water for 1 h at 60 °C, under stirring.

B) 1.20 mL of citric acid, 1.72 g sodium acetate, 2.80 mL phosphate buffer at pH 7.0, 1.00 g gelatin, and 440 mg IL (choline DHP) were blended for 1h at 60 °C, under stirring. Right before putting this solution in the electrospinning syringe, 400 μL of a 10 mg/mL solution of enzyme in phosphate buffer at pH 7.0 was added and mixed with the tip.

C) The gelatin mixture (5.5% w/w) was prepared by dissolving 233 mg of gelatin in 4.0 mL of phosphate buffer at pH 6.0, and heating at 60 °C for 1 h, under stirring. Right before putting this solution in the electrospinning syringe, 400 μL of a 20 mg/mL solution of enzyme in phosphate buffer at pH 6.0 was added and mixed with the tip.

2.5 Mixtures for preparing silica-shell/PVA core fibers

The silica shell was prepared as in section 2.2.3. The core solution was prepared according to protocol C) in section 2.4, but replacing gelatin with 706 mg of PVA solution (15%)

2.6 Mixtures for preparing films

2.6.1 Mixtures based on Ion jelly

2.6.1.1 Ionic liquid Choline DHP

To 500 μL of phosphate buffer, pH 7, were added 56 mg of the IL choline DHP and 83 mg of gelatin. The mixture was heated for 1 h at 60 °C, under magnetic stirring. When the temperature of the mixture fell down to 35 °C, 100 μL of a 1 mg/mL enzyme solution were added and well mixed with the tip. 100 μL of the fluid were placed at the bottom of a cuvette, which was kept overnight at 4 °C. A similar experiment was performed, changing the amount of gelatin to 56 mg.

2.6.1.2 Ionic liquid 1-butyl-3-methylimidazolium tetrafluoroborate

The procedure was as described for choline DHP, but [BMIM][BF_4] was added instead 56 μL.

2.6.2 Mixtures based on gelatin and ethanol

To 500 μL of phosphate buffer pH=6.0 and 500 μL of ethanol was added 167 mg of gelatin, and the mixture was heated for 1h at 60 °C ,under magnetic stirring. When the temperature of the mixture fell down to 35 °C, 100 μL of a 1 mg/mL enzyme solution were added and well mixed with the tip.

2.6.3 Mixtures based on silica and IL

A typical procedure was mixing 1000 μL of TMOS, 300 μL methanol, 245 μL of water and 13.3 μL of HCL (4 M). This solution was heated at 60 °C for min, under stirring, after which 650 mg of PVA solution at 18% were added, and the resulting mixture was

kept under stirring at 60 °C for 1 h 30 min. To increase the pH, a 1.0 M NaOH solution was added, dropwise, while the pH was successively measured. This addition, conducted under stirring, took about 30 min, after which the pH was 2.9. The mixture was allowed to cool down to 30 °C before adding 100 μL of a 1 mg/mL enzyme solution and 100 μL of the IL [$BMimBF_4$]. The fluid was spread over the bottom of a Petri dish and left to dry overnight.

2.6.4 Mixtures based on silica with ammonia

A typical sol-gel solution was prepared. To increase the pH of this solution, ammonia (0.1 M; prepared by diluting 34 μL of ammonia at 25% w/v to a final volume of 5.0 mL) was added, and pH measured. The amount of ammonia solution was selected so as to yield two different pH values: 3.5 and 5.0. Once the target value was reached, 100 μL of a 10 mg/mL enzyme solution were added. The fluid was spread over the bottom of a Petri dish and left to dry overnight.

2.7 Electrospinning conditions

2.7.1 Conventional electrospinning setup

The electrospinning power supply was a Glassman EL 30kV, connected to the metallic needle of a 4.50 cm diameter syringe that was filled with the sol-gel solution. The latter was pumped with a KDS100 pump from KD Scientific. The needle diameters can be varied, depending on the viscosity of the mixture. In this work, all mixtures were electrospun using 25G needles (0.26 mm inner diameter) from ITEC. The collector was fixed and covered with aluminum foil. The humidity and temperature of the acrylic box chamber were controlled manually, using an electric heater or using a hair dryer.

Table 2.10: Conditions for making fibers as in Table 2.5

	TMOS: H_2O: methanol: HCl: PVA	Temperature (°C)	Humidity (%)	Flow rate (mL/h)	Distance (cm)	Voltage (kV)	Deposition time (h)
1.	1.00: 2.01: 1.09: 7.85×10^{-3}: 3.45×10^{-4}	30-35	30-35	0.30	18	20	3
2.	1.00: 5.74: 1.09: 4.13×10^{-2}: 3.45×10^{-4}	26-27	40-45	0.20	20	20	0.5
3.	1.00: 5.17: 0.99: 4.13×10^{-2}: 3.45×10^{-4}	26-27	40-45	0.20	20	20	0.5
4.	1.00: 2.8: -: 4.33×10^{-4}: 2.52×10^{-8}	23-26	35-40	0.30	18	20	3
5.	1.00: 2.8: -: 4.33×10^{-4}: 7.08×10^{-8}	25-30	35-40	0.30	18	20	3

Table 2.11: Conditions for making fibers as in Table 2.7

	TEOS: Ethanol: H_2O: Citric acid: PVA	Temperature (°C)	Humidity (%)	Flow rate (mL/h)	Distance (cm)	Voltage (kV)
V)a.	1.00: 91.33: 17.26: 204.28: 2.79×10^{-3}	35-38	34-37	0.20	21	20
V)b.	1.00: 91.33: 17.26: 204.28: 4.48×10^{-3}	23-30	33-64	0.20	21	20
V)c.	1.00: 91.33: 17.26: 204.28: 5.64×10^{-3}	22-23	64-67	0.20	21	20
V)d.	1.00: 91.33: 17.26: 204.28: 8.31×10^{-3}	22-23	65-67	0.20	21	20

Table 2.12: Conditions for making fibers as in Table 2.8

	TEOS: H_2O: Ethanol: HCl: PVA	Temperature (°C)	Humidity (%)	Flow rate (mL/h)	Distance (cm)	Voltage (kV)	Deposition time (h)
1.	1.00: 2.10: 9×10^{-1}: 1.40×10^{-4}: 5.29×10^{-1}	35-38	25-28	0.20	18	20	3
2.	1.00: 2.11: 9×10^{-1}: 2.80×10^{-4}: 5.29×10^{-1}	33-35	26-28	0.20	18	20	3
3.	1.00: 2.10: 9×10^{-1}: 3.31×10^{-7}: 6.17×10^{-4}	25-30	35-40	0.30	18	20	1
4.	1.00: 2.10: 9×10^{-1}: 3.31×10^{-7}: 4.32×10^{-4}	25-30	35-40	0.30	18	20	1
5.	1.00: 8.81: 1.43: 2.80×10^{-4}: 1.40×10^{-4}	20-25	38-45	0.30	18	20	2
6.	1.00: 9.64: 1.16: 2.80×10^{-4}: 5.29×10^{-1}	20-25	38-45	0.30	18	20	2

In the case of the gelatin fibers, a 23G needle was used (0.337 mm inner diameter), due to the high viscosity of the electrospinning mixture. Assay 4 was performed with a rotating collector (at around 20 rpm) connected to a power drill.

Table 2.13: Conditions for making fibers as in table 2.9

	Temperature (°C)	Humidity (%)	Flow rate (mL/h)	Distance (cm)	Voltage (kV)	Deposition time (h)
1.	30-33	30-35	0.10-0.30	9-10	15-18	15
2.	30-33	30-35	0.10-0.30	9-10	15-18	15
3.	30-33	30-35	0.10-0.30	9-10	15-18	15
4.	30-33	30-35	0.10-0.30	9-10	15-18	45
5.	33-35	22-30	0.10-0.30	9-10	15-18	30
6.	33-35	20-30	0.10-0.30	9-10	15-18	40

Table 2.14: Conditions for making PVA fibers

	Temperature (°C)	Humidity (%)	Flow rate (mL/h)	Distance (cm)	Voltage (kV)	Deposition time (h)
18%	25-28	35-40	0.10-0.40	15	18	45
25%	25-28	35-40	0.10-0.40	15	18	30

2.7.2 Co-axial electrospinning setup

In addition to the normal setup, it is necessary to use one pump for each of the mixtures used to fabricate the shell and the core of the fibers. Different tubing materials were tested and it was found that the tubing normally used for biomedical applications (1.09 mm inner diameter) was not chemically attacked by the electrospinning solutions.

The sol-gel solution pump was placed outside of the electrospinning chamber, while the other pump was kept inside the chamber. This allowed better control of the temperature of this solution, to avoid gelation. This arrangement also required a smaller length of tubing, which was convenient because this was the solution to which enzyme was added.

The co-axial fiber block setup was from Linari Engineering, and used 2-layer Coax needles.

Table 2.15: Conditions for making silica shell/Ion jelly-core fibers

	Temperature (°C)	Humidity (%)	Flow rate (mL/h) Shell pump	Flow rate (mL/h) Core pump	Distance (cm)	Voltage (kV)	Deposition time (h)
A)	35-38	15-35	0.10-0.40	0.50-1.00	9	20	3
B)	30-34	30-38	0.10-0.30	0.60-1.00	10	20	3
C)	25-26	32-35	0.08-0.30	0.60-0.70	10	22.50	0.5

2.8 Characterization of the fibers

2.8.1 Fourier transform infrared spectroscopy (FTIR)

In this type of analysis, the spectra result from different vibration modes of the molecules of the compound. These correspond to transitions between vibrational energy states, resulting from the interaction of the compound with infrared radiation. Most molecules absorb radiation at wavenumbers between 4000 cm^{-1} and 400 cm^{-1}. The lines in the spectra refer to the vibrational modes of the molecule, corresponding to different chemical bonds [94].

The fibers obtained were analyzed by FTIR. Each sample was submitted to 32 scans between 4000 cm^{-1} and 400 cm^{-1}, using a Tensor 27 FTIR spectrometer from Bruker Optik. Data analysis was performed with 6.0 OPUS software.

2.8.2 Scanning electron microscopy (SEM)

Fiber morphology and diameter were determined by SEM. In this technique, the image is created by applying a beam of electrons or ions and scanning the response of the material [95], [96].

All SEM images were obtained using a Carl Zeiss Auriga SEM located at CENIMAT (Centro de Investigação de Materiais - Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa).

2.8.2.1 Energy Dispersive Spectroscopy (EDS)

This technique is coupled with SEM and produces chemical element profiles of regions of the material. An Oxford Instruments EDS was used.

2.8.3 Assessment of fiber membrane solubility

For testing the solubility of the fiber membranes obtained through electrospinning, different assays were performed. One consisted in adding water dropwise to the fiber membrane, deposited on a glass plate. Another consisted in submerging the membrane in water for 5 days, at room temperature. These two procedures were monitored by visual observation, focused on apparent loss of mass. To quantify weight loss, a membrane was dried at 24 h under vacuum, weighed, and immersed in water, under orbital shaking, for 2 h, after which it was again dried for 24 h under vacuum, and weighed.

2.8.4 Conductivity measurements

2.8.4.1 Films

These measurements were only performed with the gelatin-based films indicated in Table 2.16. Measurements were done in triplicate. The sol-gel formulation was deposited on a warm (45 °C) glass plate, to avoid immediate solidification and allow the spreading of the liquid over the plate. After 15 min, the film obtained was sliced in equal strips.

Table 2.16: Solutions for conductivity tests

	Gelatin	Choline DHP (mg)	Water (μL)
1)	17.39	17.39	200
2)	22.22	22.22	200
3)	90.00	45.00	200
4)	160.00	40.00	200
5)	94.12	40.00	200

2.8.4.2 Membranes

These measurements were with membranes prepared with solution 1 (Table 2.5).

2.9 Enzyme activity measurements

2.9.1 Enzyme activity standard assay in aqueous medium

The enzyme used in these studies was HRP, an oxidoreductase belonging to class E.C. 1.11.1.7, and acting on peroxide as acceptor. The enzyme was allowed to warm up to room temperature before being used.

Enzyme solutions were prepared with 100 mM phosphate buffer at pH 7.0, obtained by mixing 19.50 mL of solution A with 30.50 mL of solution B, and adjusting the final volume to 100 mL. Solution A was a 0.20 M solution of sodium phosphate monobasic, prepared by dissolving 1.38 g of sodium phosphate monobasic monohydrate in water, under magnetic stirring for 20 min, and adjusting the final volume to 50 mL. Solution B was a 0.20 M solution of di-sodium hydrogen phosphate, prepared by dissolving 1.42 g of di-sodium hydrogen phosphate dihydrate in water, under magnetic stirring for 20 min, and adjusting the final volume to 50 mL.

To obtain phosphate buffer at pH 6.0, 7 mL of solution A were added to 1 mL of phosphate buffer at pH 7.0.

An approximately 100 mM PSA solution was prepared by dissolving 46.4 mg of sodium 4-hydroxybenzenesulfonate dihydrate in 2.0 mL of water, under magnetic stirring, for 15 min, at room temperature. An approximately 0.16 mM 4-AAP solution was prepared by first dissolving 4.0 mg of 4-aminoantipyrine in 1.0 mL of water, and diluting 125 times. An approximately 5 mM H_2O_2 solution was prepared by mixing 2.5 μL of hydrogen peroxide with 5.0 mL of water. An approximately 1 mg/mL enzyme solution was prepared by

dissolving 2 mg of HRP in 1 mL of phosphate buffer, and diluting 2 times.

All solutions were prepared fresh for enzymatic assays.

The method used to quantify enzyme activity was UV/VIS Spectroscopy at 490 nm, based on the monitoring of the formation of a pink colored dye as following demonstrate in equation (2.1):

$$2H_2O_2 + PSA + 4 - AAP \rightarrow \text{quinoneimine dye} + 3H_2O + NaHSO_4 \qquad (2.1)$$

A typical assay was performed by mixing in a 1 mL cuvette 500 μL of phosphate buffer, 250 μL of PSA solution, 250 μL of 4-AAP solution, and 63 μL of enzyme solution (1mg/mL), and setting zero absorbance. Then 10 μL of H_2O_2 solution were added, well mixed with the tip, and the increase in absorbance was monitored for 60 s.

2.9.2 Enzyme activity assay with IL in aqueous medium

In these assays, 119 mg of Choline DHP were added to 500 μL of phosphate buffer, under magnetic stirring, at room temperature. Then 250 μL of PSA solution, 250 μL of 4-AAP solution, and 63 μL of enzyme solution were added, and zero absorbance was set. Upon addition of 10 μL of H_2O_2 solution, the increase in absorbance was monitored for 60 s.

2.9.3 Enzyme activity assay with acetate buffer

To evaluate the effect of high ionic strength on enzyme activity, to 500 μL of acetic acid, glacial, were added 0.717 g of sodium acetate trihydrate, and the mixture was stirred at 60 °C, for 20 min. The concentration of the acetate buffer thus prepared was approximately 17 M, and its pH was 4.5 (pKa = 4.76 for acetic acid). When the buffer cooled down to room temperature, it was put in a cuvette, to which were added 250 μL of PSA solution, 250 μL of 4-AAP solution, and 63 μL of enzyme solution. Zero absorbance was set. Upon addition of 10 μL of H_2O_2 solution, the increase in absorbance was monitored for 60 s.

A similar experiment was performed with acetate buffer, prepared from acetate buffer ≈17 M diluted 50 times, to give a concentration of approximately 340 mM.

2.9.4 Enzyme activity assay with acetate buffer and gelatin

To 500 μL of acetate buffer, ≈17 M, were added 119 mg of gelatin, and the mixture was stirred at 60 °C, for 30 min. The following procedure was as in 2.9.3.

2.9.5 Activity of enzyme immobilized in Ion jelly films

The films were prepared as described in 2.5.1. The next day, to the cuvette were added 500 μL of phosphate buffer at pH 7, 125 μL of PSA solution, 125 μL of 4-AAP solution, and 63 μL of H_2O_2 10 mM (prepared by mixing 5 μL of H_2O_2 at 30% w/v with 5.0 mL of water).

2.9.6 Activity of enzyme immobilized in silica films with IL

The films were prepared as described in 2.5.2. The next day, to the Petri dish were added 500 μL of phosphate buffer at pH 6, 250 μL of PSA solution, 250 μL of 4-AAP solution, and 100 μL of H_2O_2 5 mM.

2.9.7 Activity of enzyme immobilized in fiber membranes

Enzymatic assays were performed in Petri dishes, and were only qualitative, based on visual observation.

Chapter 3

Results and Discussion

For clarity in the discussion of the results obtained, the Tables shown in section 2. are reproduced here.

3.1 Development of sol-gel solutions for testing in electrospinning

Throughout the text, when the term "good viscosity" is used to characterize a sol-gel solution, it means that the viscosity of that solution is adequate for electrospinning. Large surface area, high permeability and small pore size are characteristics that can be obtained in a membrane fabricated by electrospinning using the silica sol-gel process [81]. In spite of the apparent simplicity of the chemistry beyond the sol-gel process (equations (1.1)-(1.3)), the characterization of reaction mechanisms and species formed in each step is difficult, which makes it extremely hard to control the full process. Although the sol-gel process comprises two distinct phases, namely the - *sol* – a colloid that is able to generate, through inorganic polymerization, a tridimensional hard structure – *the gel* – the coexistence of the two phases is a common situation.

3.1.1 TMOS-based sol-gel solutions without PVA

The first part of this thesis consisted in studying different molar ratios and ways to do the sol-gel solutions, in order to understand the influence of the solution viscosity and the timeframe available before a solid structure formed. Also the solids obtained were observed and physical characteristics such as rigidity, and apparent tensile strength were determined.

The many sol-gel solutions prepared were carefully documented as regards several parameters known to affect the process, such as temperature, stirring, time and order of addition of

components, type of solvent used in addition to silica, water and acid, and the molar ratios of the components. A main concern with sol-gel solutions to be used in electrospinning is the time it takes before the mixtures solidify.

Table 3.1: Molar ratios used for preparing sol-gel solutions without PVA (Table 2.1)

	TMOS	H_2O	HCl	Comments
a)	1.00	10.01 (1.82+8.19)	8.26×10^{-5}	Became solid after about 12 h
b)	1.00	10.01	8.26×10^{-5}	Became solid after about 12 h but had a softer texture than (a)
c)	1.00	10.01	3.30×10^{-4}	Was still a gel after 15 h
d)	1.00	22.93	8.26×10^{-5}	Became solid after 15h. Broke into small crumbs
e)	1.00	10.01 (1.82+8.19)	8.26×10^{-5}	Became solid after 6 h
f)	1.00	10.01	8.26×10^{-5}	Became solid after 6 h but had a softer texture than e)
g)	1.00	10.01	8.26×10^{-4}	Became solid after about 13 h
h)	1.00	1.82	8.26×10^{-4}	Became solid after 5 h became solid. Broke into large pieces.
i)	1.00	4.09	7.38×10^{-3}	Became solid after 24 h, but some liquid remained
j)	1.00	1.82	8.26×10^{-5}	Became solid after 4 h. Broke into large pieces.
k)	1.00	16.38	8.26×10^{-5}	Became solid after 14 h. Broke into small crumbs

It was found (Table 3.1) that the higher the amount of HCl within the range tested, the longer the sol-gel solution remained fluid. Also higher amounts of acid made the texture of the sol-gel tougher, and when it broke, it broke into large pieces. On the other hand, with small amounts of acid, as in case j), the sol-gel solution became solid faster, and the sol-gel obtained was more crumbly and amenable to break into small pieces, almost like a powder.

The properties of the sol and gel phases are highly influenced by the reactions of hydrolysis and condensation (equations (1.2) and (1.3)). The rate of hydrolysis is directly proportional to the concentration of the species catalyzing the reaction, which in this case is H_3O^+ from HCl (Figure 3.1), and decreases as pH increases up to neutral. The rate of condensation depends strongly on how pH compares with the isoelectric point of silica (pH in the range 2-3). Below this point, condensation is catalyzed by H_3O^+ ions. The polymerization process involves reactions between neutral species and protonated silanol groups on monomers or at terminal positions on oligomers, leading to linear chains or branched chains with a low degree of ramification. These grow into highly branched but low degree of condensation structures, with a low concentration of siloxane bonds.

Above the isoelectric point of silica, the polymerization process is catalyzed by hydroxyl groups and involves the deprotonation of silanol groups. The growth of polymeric structures occurs preferentially by the addition of smaller species to more highly condensed ones, resulting in discrete aggregates with many cross link. Due to the high number of siloxane bonds per unit volume, and therefore a high degree of condensation, the structures obtained are more compact. Since the solubility of silica is low at low pH, the aggregates formed are not bigger than 2-4 mm. [39]

The pH of the formulations listed in Table 3.1 was between 3 and 4, which should lead to a high concentration of silanol groups per unit volume unit, resulting in compact sol-gel structures, as observed by visual inspection.

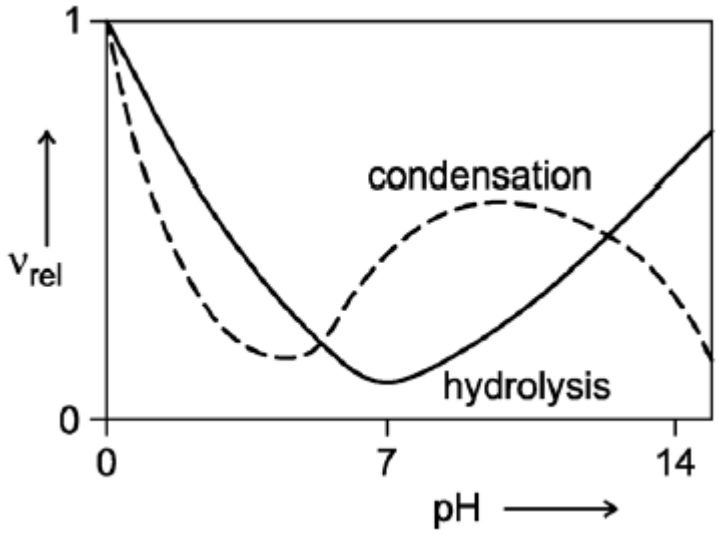


Figure 3.1: Influence of the pH and the rate of hydrolysis and condensation for forming sol-gel structures [97]

3.1.2 TMOS-based sol-gel solutions with PVA

To understand the influence of the addition of PVA, several experiments were performed.

Table 3.2: Molar ratios used for preparing sol-gel solutions with PVA – I (Table 2.2)

	TMOS	H_2O	HCl	PVA	Comments	Comments
l)	1.00	10.01	8.26×10^{-5}	8.54×10^{-5}		Became solid in 2 h after PVA was added.
m)	1.00	10.01	8.26×10^{-5}	7.64×10^{-2}		Became solid in less than 5 min after PVA was added.
n)	1.00	10.01	8.26×10^{-5}	7.64×10^{-2}	The sol-gel solution was not heated	Became solid in 1 h after PVA was added.
o)	1.00	5.73	4.43×10^{-3}	7.64×10^{-5}	The sol-gel solution was heated at 58 °C for 35 min	After 2 h 30 min the consistence was still good.
p)	1.00	5.73	4.43×10^{-3}	2.70×10^{-4}	The sol-gel solution was heated at 55 °C for 1 h 15 min	The viscosity started to increase after 40 min, stabilized and was maintained for ≈24 h
q)	1.00	5.73	4.43×10^{-3}	4.57×10^{-5}	The sol-gel solution was heated at 55 °C for 1 h 15 min	The viscosity started to increase after 20 min, stabilized and was maintained for ≈24 h
r)	1.00	5.73	1.48×10^{-3}	-	The sol-gel solution was heated at 63 °C	Became solid in 40 min
s)	1.00	5.73	4.43×10^{-3}	-	The sol-gel solution was heated at 50 °C for 2 h	The gel consistency was maintained for at least 24 h
t)	1.00	5.73	2.95×10^{-3}	-	The sol-gel solution was heated at 62 °C	Became solid in 50 min
u)	1.00	5.73	4.43×10^{-3}	-	The sol-gel solution was heated at 58 °C	Became solid in 55 min

Table 3.2 allowed looking at the influence of the PVA content and temperature of the sol-gel solution on the solidification time. At temperatures around 50 °C, the sol-gel remained fluid for a longer time. Temperature also influenced the sol-gel texture, increasing temperatures making the sol-gel matrix easier to break into pieces. Increasing amounts of PVA solution made the sol-gel solution solidify faster, and the resulting sol-gel matrix was easier to break into small and soft pieces. This can be explained by the presence of partially hydrolyzed acetate groups. The main mechanism involved is the interaction between the hydroxyl groups of PVA and the hydroxyl groups of silanol, from hydrolyzed silica (Figure 3.2), which can promote faster condensation.

The knowledge acquired from the previous experiments allowed to conduct a study focused on the influence of temperature on the sol-gel process. Three temperatures were tested, namely room temperature for reference, 50 °C, which brought along a pronounced increase

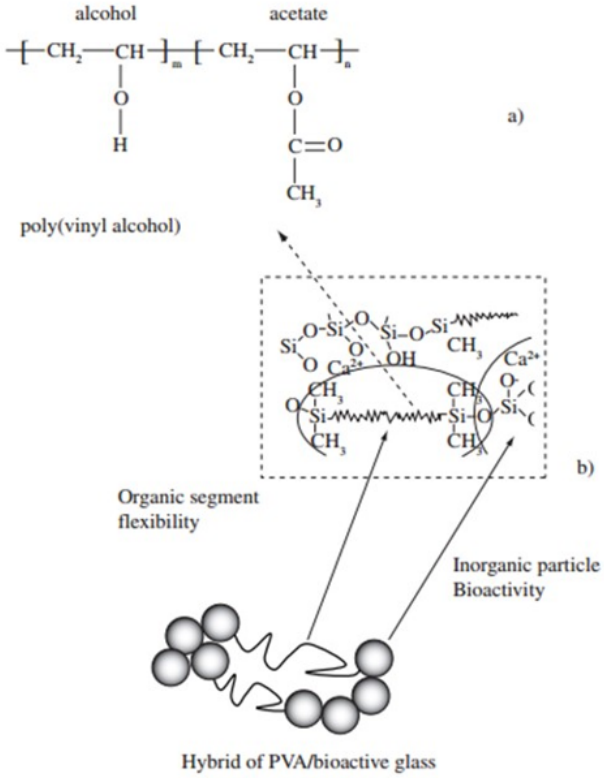


Figure 3.2: Schematic drawing of the inorganic-organic hybrid synthesized based on PVA polymer and bioactive glasses; a) PVA chain with functional groups; b) Hybrid network structure after reaction with temperature.[94]

in the time it took for the sol-gel solution to solidify – always a main concern in this thesis – and 60 °C, which is widely reported in the literature.

After solidification, matrices obtained from solutions 1.a) 1.c) 1.d) 3.c) 3.d) broke into big pieces, while those from solution 1.c) seemed to have water around them. Matrices from solutions 1.b), 3.a) and 3.b) broke into small, soft, pieces (slices). Solution 8 led to tougher sol-gel matrices, present in Table 3.3.

Higher temperature, and longer times of preparation led to harder matrices. Lower amounts of HCl had the same effect. This can be explained by higher pH values, which favor fewer nucleation centers more prone to form monomer-agglomerate species, leading to a lower amount of particles, although of larger size.

The water/alkoxy group molar ratio (R) is one of the most important parameters in the sol-gel process, with a strong influence on the time of gelation, due to the fact that water contributes simultaneously to the hydrolysis reaction, as a reagent, and to the condensation reaction, of which it is a product. For complete reaction of the silica precursors, it is necessary to have two mol of water per mol of TMOS or TEOS. To evaluate the influence

Table 3.3: Molar ratios used for preparing sol-gel solutions with PVA – II (Table 2.3)

	TMOS	H_2O	HCl	PVA	Temperature of mixing step (1 h)	Comments
1.a)	1.00	5.73	1.48×10^{-3}	1.38×10^{-4}	50 °C	After 45 min the viscosity started to increase. Started to solidify after 5 h.
1.b)	1.00	5.73	1.48×10^{-3}	2.06×10^{-4}	50 °C	After 40 min the viscosity started to increase.
1.c)	1.00	5.73	1.48×10^{-3}	1.97×10^{-4}	50 °C	After 20 min the viscosity started to increase. Started to solidify after 30 min.
1.d)	1.00	5.73	1.48×10^{-3}	2.94×10^{-4}	50 °C	After 20 min the viscosity started to increase. Started to solidify after 25 min.
2.a)	1.00	5.73	1.48×10^{-3}	1.38×10^{-4}	60 °C	After 30 min the viscosity started to increase. Started to solidify after 3 h.
2.b)	1.00	5.73	1.48×10^{-3}	2.06×10^{-4}	60 °C	After 30 min the viscosity started to increase. Started to solidify after 4 h.
3.a)	1.00	5.73	4.43×10^{-3}	1.38×10^{-4}	50 °C	After 45 minutes the viscosity starts increasing. After 3h the viscosity was good
3.b)	1.00	5.73	4.43×10^{-3}	2.06×10^{-4}	50 °C	After 35 min the viscosity started to increase. After 3 h the viscosity was good.
3.c)	1.00	5.73	4.43×10^{-3}	1.97×10^{-4}	50 °C	After 10 min the viscosity started to increase. Started to solidify after 25 min.
3.d)	1.00	5.73	4.43×10^{-3}	2.94×10^{-4}	50 °C	After 10 min the viscosity started to increase. Started to solidify after 20 min.
4.a)	1.00	4.09	7.38×10^{-4}	4.54×10^{-4}	50 °C or room temperature	After 2 h at room temperature the solutions remained unchanged. Started to solidify after 18 h.
4.b)	1.00	4.09	7.38×10^{-4}	3.63×10^{-4}	50 °C or room temperature	After 2 h at room temperature the solutions remained unchanged. Started to solidify after 30 h.
5.a)	1.00	4.09	7.38×10^{-4}	6.81×10^{-4}	50 °C or room temperature	After 2 h at room temperature the solutions remained unchanged. Started to solidify after 30 h.
5.b)	1.00	4.09	7.38×10^{-4}	5.45×10^{-4}	50 °C or room temperature	After 2 h at room temperature the solutions remained unchanged. Started to solidify after 30 h.
6	1.00	4.79	1.25×10^{-3}	-	60 °C	After 2 h at room temperature the solutions remained unchanged. Started to solidify after 18 h.
7	1.00	5.73	4.43×10^{-3}	-	60 °C	After 2 h at room temperature the solutions remained unchanged. Started to solidify after 16 h.
8	1.00	4.79	1.25×10^{-3}	-	50 °C	After 2 h at room temperature the solutions remained unchanged. Started to solidify after 26 h.

of R, lower amounts of water were used for the same amount of HCl (Table 3.3)

These sol-gel solutions were tested in electrospinning experiments (Table 3.4). The biggest obstacle was to find a suitable viscosity of the sol-gel solution. The addition of the PVA solution was made before filling the syringe.

When the viscosity was optimized, the time available for fiber production was very low (5

Table 3.4: Molar ratios used for preparing sol-gel solutions with PVA – III (Table 2.4)

	TMOS	H_2O	HCl	PVA	Notes
i)	1.00	5.73	1.48×10^{-3}	1.06×10^{-1}	-
ii)	1.00	5.73	1.48×10^{-3}	6.64×10^{-2}	-
iii)	1.00	5.73	1.48×10^{-3}	8.85×10^{-2} $(6.64 \times 10^{2} + 2.21 \times 10^{-2})$	2 additions, 20 min apart
iv)	1.00	5.73	1.48×10^{-3}	6.64×10^{-2}	Sol-gel solution prepared at 45 °C for 45 min
v)	1.00	5.73	1.48×10^{-3}	8.85×10^{-2} $(6.64 \times 10^{2} + 2.21 \times 10^{-2})$	Sol-gel solution prepared at 45 °C for 45 min
vi)	1.00	5.73	1.48×10^{-3}	15.92×10^{-5} $(7.96 \times 10^{-5} + 7.96 \times 10^{-5})$	Sol-gel solution prepared at 60 °C for 50 min. 2 additions, 20 min apart
vii)	1.00	5.83	1.50×10^{-3}	2.48×10^{-4}	-
viii)	1.00	5.83	1.50×10^{-3}	3.74×10^{-4}	-
ix)	1.00	5.83	1.50×10^{-3}	4.66×10^{-4}	-
x)	1.00	5.83	1.50×10^{-3}	5.63×10^{-4}	-

min, at the most), which makes this approach unsuitable for producing membranes. The best formulation was i). In spite of the high amounts of PVA, this sol-gel solution had good viscosity, before it solidified.

A way to circumvent this problem might have been to decrease the time between mixing the silica and the PVA solutions, as done by Tong and co-workers. [81] However, this method for making sol-gel matrices with PVA was abandoned given the high risk of solidification inside the electrospinning apparatus. Even when the time window for making fibers is optimized, parameters such as modification of contact surface may lead to faster solidification. The biggest concern was the possibility of solidification inside coaxial needles.

As regards cleaning/removing sol-gel solutions, it was found that among water, acetone and ethanol, ethanol was the best solvent, although for removing sol-gel residues it was necessary to scrape the surface of the container for full cleaning.

3.1.3 TMOS-based sol-gel solutions with PVA and methanol

As referred earlier, there are many variables that influence the sol-gel process. Small changes in these variables can produce huge changes in the materials obtained. Finding

the right balance of the molar ratios of the species involved was essential to create a window of opportunity for doing electrospinning.

The formulations with methanol led to structures resembling cotton-wool (Figure 3.3). In comparison with a 2D membrane, those structures have the advantages of increased length of fibers and mainly, a gain in surface area. 3D structures such as this have been finding application in different areas, such as tissue engineering, solar cells, and with particular relevance for this work, filtration. [98], [99]. These structures can be obtained using mainly four strategies: (1) By increasing the electrospinning time; (2) By assembling the 3D membrane from the 2D membrane through layer-by-layer electrospinning, sintering and mechanical expansion; (3) By assembling the membrane with a 3D or liquid collector; (4) Through self-assembly, which was the case here. This was achieved just by controlling the temperature, humidity and viscosity of the sol-gel solution. [98]

(a) Distance between colector and the top of the fiber mat was 3.0 cm

(b) Distance between colector and the top of the fiber mat was 5.8 cm

Figure 3.3: Cotton-wool-like membranes.

3.1.4 TMOS-based sol-gel solutions with PVA and acetic acid

Oriero and co-workers [92] studied the production of silica fibers through the sol-gel process via electrospinning. The authors reported on the production of fibers without deposition,

which allows the formation of a membrane, and mentioned that the maximum time allowed by their experimental conditions was 35 min. Looking into detail at the molar ratios used, the concentration of HCl did not seem high enough to ensure fluidity of the sol-gel solution long enough to make fibers in our apparatus. Even so, their protocol was tested.

Looking at the literature available, the molecular weight and hydrolysis degree of PVA are important parameters in the process , namely on the time before solidification. In this case, a different PVA was used, in different concentrations, but the formulations obtained were not adequate for electrospinning. As already mentioned, through hydrogen bonding between hydroxyl groups from PVA and from the siloxane network, faster condensation of the silica structure may occur.

The addition of acetic acid caused a delay in the solidification of the sol-gel solution (solutions 4 and 5 - Table 3.5), as reported.

Table 3.5: Molar ratios used for preparing sol-gel solutions with PVA and acetic acid

	TEOS	H_2O	HCL	Acetic acid	PVA	Comments
1.	1.00	2.00	2.41×10^{-4}	-	3.24×10^{-6}	When an aqueous solution of PVA was added the sol-gel solution became solid.
2.	1.00	2.00	2.41×10^{-4}	-	2.52×10^{-6}	When an aqueous solution of PVA was added the sol-gel solution became solid.
3.	1.00	2.00	2.41×10^{-4}	-	1.80×10^{-3}	When an aqueous solution of PVA was added the sol-gel solution became solid.
4.	1.00	2.00	2.41×10^{-4}	3.01×10^{-4}	2.52×10^{-3}	The solution of acetic acid was added simultaneously with that of PVA. The solution became solid after being 10 min in the electrospinning plastic syringe.
5.	1.00	2.00	2.41×10^{-4}	6.02×10^{-4}	7.08×10^{-3}	The solution of acetic acid was added simultaneously with that of PVA. The solution became solid after being 12 min in the electrospinning plastic syringe.

3.1.5 TEOS-based sol-gel solutions with PVA, ethanol and citric acid

The main concern being the time window before solidification of the sol-gel solution, a new approach was followed, based on a protocol for producing bioglass, which was reported to fulfill the above requirement. This protocol was adapted for producing sol-gel, using a silica precursor, water, an acid and a solvent, and including addition of a PVA aqueous solution [3.6].

In spite of the changes to the original protocol, the sol-gel solution remained fluid for a long time (over 1 week). This seemed like a remarkable progress and the next step was optimizing the electrospinning conditions and produce fibers.

Table 3.6: Molar ratios used for preparing sol-gel solutions with PVA, ethanol and citric acid (Table 2.7)

	TMOS	H_2O	Ethanol	Citric acid	PVA	Notes
I))	1.00	410.08	17.26	51.07	-	
II))	1.00	410.08	17.26	51.07	-	After adding citric acid, the sol-gel solution was heated for 1 h at 60 °C (*)
III))	1.00	410.08	17.26	51.07	3.11×10^{-2}	
IV)	1.00	100.50	17.26	-	-	Prepared with HCl instead of citric acid
V)	1.00	91.33	17.26	204.28	-	
II)a.)	1.00	91.33	17.26	51.07	2.79×10^{-3}	(*)
II)b.)	1.00	91.33	17.26	51.07	4.48×10^{-3}	(*)
II)c.)	1.00	91.33	17.26	51.07	5.64×10^{-3}	(*)
II)d.)	1.00	91.33	17.26	51.07	8.31×10^{-3}	(*)
IV)a.)	1.00	84.92	17.26	-	2.79×10^{-3}	Prepared with HCl instead of citric acid
IV)b.)	1.00	84.92	17.26	-	4.48×10^{-3}	Prepared with HCl instead of citric acid
IV)c.)	1.00	84.92	17.26	-	5.64×10^{-3}	Prepared with HCl instead of citric acid
V)a.)	1.00	91.33	17.26	204.28	2.79×10^{-3}	
V)b.)	1.00	91.33	17.26	204.28	4.48×10^{-3}	
V)c.)	1.00	91.33	17.26	204.28	5.64×10^{-3}	
V)d.)	1.00	91.33	17.26	204.28	8.31×10^{-3}	

Solutions [I)-IV] from Table 3.6 just produced electrospray, even when the amount of PVA solution was increased.

The main problem with this protocol was the use of large amounts of water. The rate of the hydrolysis reaction increases with the increase in R, as expected, but for R values much higher than 4 the diffusion of the water molecules no longer controls the process which becomes kinetically controlled. R values higher than 4 also promote the hydrolysis of siloxane groups, resulting in lower rates of the condensation reaction. Very high R values can additionally lead to the phase separation of the silica/water mixture.[39]

High amounts of water can make solidification faster up to 1000 times, but this effect can be counteracted by increasing the amount of acid. In addition to its effect on gelation, the value of R also affects the sol-gel structural parameters, such as porosity. [100]

3.2 TEOS-based sol-gel solutions with PVA and ethanol

In order to adjust the ratios of each species in the sol-gel solution, and how they affected the quality of the fibers produced, additional experiments were performed (Table 2.8). The solvent may improve the mixing of TEOS and water, as well as affect the reactions of hydrolysis and condensation. This is influenced by the polarity of the solvent and the presence of labile protons. Ethanol has the ability to establish hydrogen bonds, and consequently, to affect the solvation of the species involved in the sol-gel process. A large amount of alcohol can decrease the rate of condensation processes by breaking the siloxane bonds through alcoholysis (the reverse of equation (3.1)), or by replacement of the hydroxyl group in silanol by an –OR group [101].

$$\equiv Si-OH+(C_2H_5O)_3Si(C_2H_5) \underset{\text{Alcoholysis}}{\overset{\text{Alcohol condensation}}{\rightleftharpoons}} \equiv Si-O-Si(C_2H_5)+EtOH \quad (3.1)$$

Since water acts as nucleophile, the balance between alcohol and water should be carefully controlled to extend the time that the sol-gel solution remains fluid and spinnable. As seen earlier, below the isoelectric point of silica, the rate of polymerization decreases, due to the fact that the silicon atom becomes more electrophilic and amenable to attack by silanol groups, releasing water. This does not favor aggregation or formation of aggregates with a high degree condensation. This must have been the case of sol-gel solutions (1-4), whose pH was below 1. The sol-gel process have a vast of variables that the influence the solution produced. Vary into a narrow limit leads a huge changes. The equilibrium of molar rations was fundamental to find a window of opportunity that permit do electrospinning.

3.3 Development of mixtures without silica for testing electrospinning

For testing conditions for producing the core of coaxial fibers, several experiments were performed, varying the amounts water or phosphate buffer, and gelatin.

3.3.1 Mixtures based on Ion jelly

The sol-gel solution tested first was solution 6 in Table 2.9, which has just IL, gelatin and water, which are the components of a typical Ion jelly material. Although such combination

of components was shown to support enzyme activity, it presented a great problem for electrospinning which was gelation below 35 °C. Since the electrospinning chamber was not set to work at this temperature, it was necessary to heat the syringe directly. In this case, a hair dryer was used. A major concern was controlling the temperature of the syringe to prevent the denaturation of the enzyme. HRP is reported to function best at 35 °C, and above it, it activity decreases.[36] [33] Using the hair dryer made it difficult to keep the humidity in the chamber above 15%, a value below which the manufacturing of silica fibers is compromised.

Most of the sol-gel solutions listed above had acetic acid in order to maintain adequate viscosity for electrospinning and avoid solidification at temperatures below 30 °C. Assays were conducted to find out the minimum amount of acid that was effective in that respect, starting with acid acetic/water ratios of 70/30. A major problem with the use of acetic acid was the pH of this solution, which was around 1, thereby compromising experiments with enzyme. The approach was adequate for forming fibers, and thus the concern now was the low pH. This led to the addition of sodium acetate, to make a buffer at pH for 4.8. All the solutions with sodium acetate produced fibers. But those with less gelatin led to fibers that were water soluble (formulation 3), and this condition was not desired. Therefore, the following studies were performed with solution 4.

3.3.2 Mixtures based on Ion jelly, with enzyme

There were several goals to achieve: the enzyme must stay in the fibers (must not leach), the fibers must me permeable to solutes, and diffusion limitations should not be excessive. After suspending the Ion jelly fiber membrane in phosphate buffer at an adequate pH for enzyme activity, a standard enzymatic assay was performed. This assay revealed no activity. The system was then heated in an orbital shaker at 35 °C. This time enzyme activity tested positive, but on the other hand the membrane started to dissolve. Therefore this assay only allowed to establish that even after being immobilized at conditions of high ionic strength, imparted by the concentrations of acetic acid and acetate, the membrane provided an encapsulation environment that did not irreversibly inactivate the enzyme. Dissolution of the membrane at room temperature was not expected. One characteristic aspect of the Ion jelly supports reported in the literature is their water insolubility and temperature resistance. This condition was not observed, either because of interference of the acetate buffer, insufficient time for promoting gelatin-IL interactions upon preparing the Ion jelly, or the fact that the IL used had not been tested before for this purpose and the protocol applied was not the most adequate.

3.3.3 Mixtures based on PVA

PVA fibers were successfully manufactured, but they dissolved fast in water. This was not a desired characteristic in this work, although such fibers may be advantageously used in applications requiring such type of scaffold.

3.4 Mixtures for electrospinning preparing silica-shell/Ion jelly-core fibers

As concerns approaches A) – producing Ion jelly fibers – and B) – producing Ion jelly fibers in the presence of acetate buffer of high ionic strength, the problems encountered were already mentioned. In addition, when using acetate buffer electrospinning did not lead to continuous fibers due to the formation of drops at the end of the Taylor cone. To try and overcome this problem the flow rate of the shell and core solutions was decreased. However, with this change in conditions the formation of fibers decreased too. A higher voltage would be required to maintain a continuous jet. Another problem was the fact that the core solution had electrical conductivity, making it difficult to maintain electrospinning conditions stable. In the case of approach C), the concentration of gelatin was lowered to increase time before gelation. However, the viscosity of the solution was too low, and drops formed at the tip of the needle and fell down, making it impossible to achieve encapsulation by silica. Fibers thus obtained also tested negative for enzyme activity.

3.5 Mixtures for preparing silica-shell/PVA core fibers

In order to achieve a better core for making coaxial fibers, PVA was tested. In this case, the good viscosity imparted by PVA helped in the manufacturing of the fibers, without losing solution dropwise after forming the Taylor cone. The membrane obtained tested negative for enzyme activity. But this may be due to the low amounts of enzyme contained in the membrane. Due to the difficulty in stabilizing the electrospinning steup, the time of deposition was low, and the amount of enzyme in the membrane must not exceed 0.025 mg.

3.6 Mixtures for preparing films

3.6.1 Mixtures based on Ion jelly

3.6.1.1 IL choline DHP

To study the activity of the enzyme in Ion jelly-based fiber cores, two films were made with different amounts of gelatin. This affected the time it took for the pink color, an indication of enzyme activity, to appear. When assaying the film with a higher amount of gelatin, the pink color took longer to appear, which suggests diffusional problems. But still, the two films led to similar values of absorvance, indicating similar catalytic ability of the enzyme.

Figure 3.4: Cuvettes with Ion jelly films at the bottom, containing HRP, with the characteristic pink color that indicates the entrapped enzyme is active

3.6.1.2 IL [BMIM][BF_4]

Similar experiments were performed, replacing choline DHP with [BMIM][BF_4]. These films also tested positive for enzyme activity.

3.6.2 Mixtures based on gelatin and ethanol

In order to delay gelation and increase the time available for electrospinning, ethanol was added to the preparation. Enzyme assays were done as above, but no color appeared, suggesting that the presence of ethanol had a negative impact on the enzyme microenvironment.

3.6.3 Mixtures based on silica and IL with added base

To increase the pH of the silica solution so as to be able to incorporate enzyme in it while avoiding enzyme denaturation, and produce fibers with entrapped enzyme, an NaOH solution was added, which changed pH to around 2.8. The IL [BMIM][BF_4] was added as well, to help create an ionic microenvironment. The film produced had a yellow color, imparted by this IL. After testing for enzyme activity, the characteristic pink color was observed, indicating that the enzyme remained active.

(a) (b)

Figure 3.5: Images of fragmented silica films with immobilized HRP, which tested positive for enzyme activity, as evidenced by the pink color developed upon adding the test solutions

3.6.4 Mixtures based on silica with added base

With the same purpose as above, films were prepared from a sol-gel solution whose pH was increased to 3.5 or 5.0 through the addition of ammonia solution [82]. Both films revealed enzyme activity, which suggests that the use of ammonia may be a good strategy to pursue.

3.7 Characterization of the fibers

3.7.1 Fourier transform infrared spectroscopy (FTIR)

Through FTIR analysis it is possible to determine which chemical functional groups are present in the membranes produced.

3.7.1.1 Mixtures based on PVA

The spectrum of PVA presents a broad band around 3600-3100 cm^{-1} assigned to O-H stretching from strong hydrogen bonding water. In the same range of frequencies, around 3300 cm^{-1}, can be seen the band corresponding to the hydroxyl group of fully hydrolyzed PVA. The peak at 2950-2850 cm^{-1} is assigned to C-H alkyl stretching. The other peaks with similar intensity near 1700 cm^{-1} and 1150-1090 cm^{-1} can be attributed toof C=O and C-O bands corresponding to the remaining non-hydrolyzed vinyl acetate (PVA is obtained through hydrolysis of polyvinylacetate).[94]

3.7.1.2 Fibers based on TMOS sol-gel

The results described below refer to the membrane obtained from solution 1 in Table 2.5. The other sol-gel solutions led to fiber membranes with similar spectra that are shown in the attachments. The broad bands around 3400-3000 cm^{-1} and 1635.22 cm^{-1} correspond to the stretching and bending of the O-H off water, respectively. The strong peaks at 1062.06 cm^{-1} and also at 1200 cm^{-1} are assigned to the Si-O-Si asymmetric bending vibration. In the range of 1500-900 cm^{-1} several bands overlap due to the presence of PVA in the sol-gel solutions. Symmetric vibrational beinding modes of Si-O-Si give rise to bands at 790 cm^{-1} and 450 cm^{-1}. The 950 cm^{-1} band is attributed to silanol groups (SI-OH stretching).

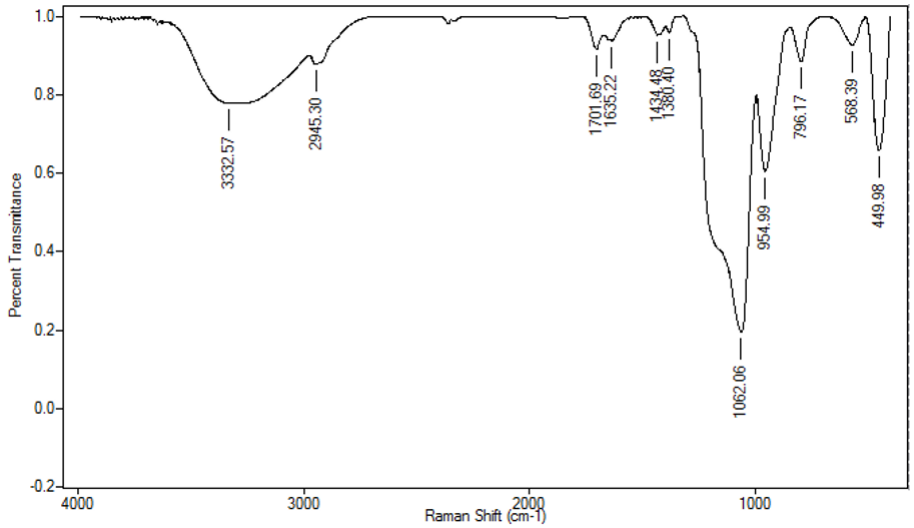


Figure 3.6: FTIR spectrum of a TMOS-based fiber membrane obtained from solution 1 in Table 2.5

3.7.1.3 Fibers based on TEOS sol-gel

Silica and ethanol have similar vibrational modes and therefore can yield overlapping peaks. [102]

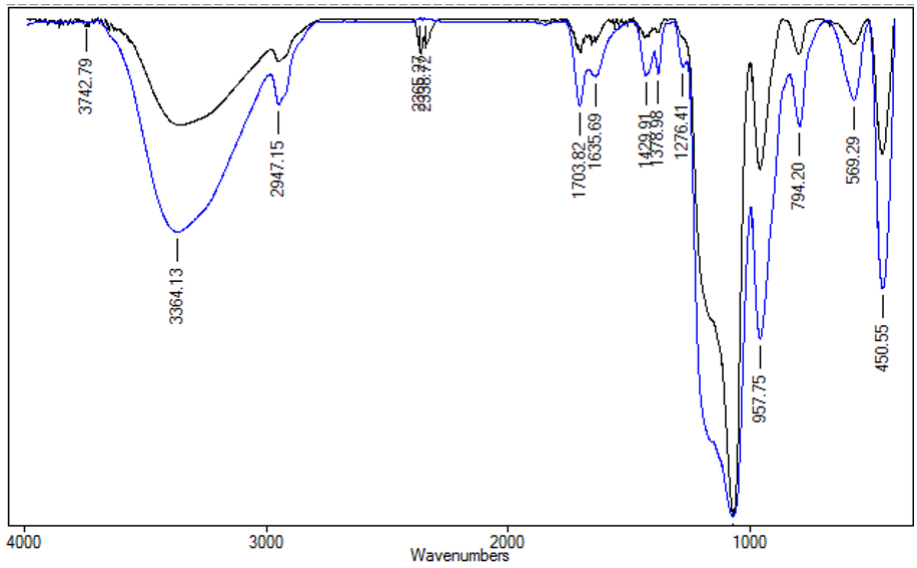


Figure 3.7: FTIR spectrum of a TEOS-based fiber membrane obtained from solution 1 and solution 2 in Table 2.8, respectively. Blue and black lines for lower and higher amounts of HCl, respectively.

In the range from 900 cm^{-1} to 1300 cm^{-1}, it is normal to have Si-alkoxyl and siloxane bands overlap. TEOS shows strongest absorption at 1100-1075 cm^{-1}, and less pronounced peaks in the range of frequencies 1170-1160 and 970-940 cm^{-1}. This latter range can result from both Si-OH and Si-O- stretching modes. Unreacted TEOS also contributes to this band, which precludes clear assignment of the bands corresponding to unreacted alkoxyl groups.

Even so, it is possible to conclude that there exist residual silica cyclic structures, due to the sol-gel process, with characteristic vibration frequencies in the range 550-640 cm^{-1}, and a main band at around 1300-1000 cm^{-1}. The Si-O-C band also overlap in this range of frequencies. The bands for water normally appear in ranges around 3500-3300 cm^{-1} (OH-H stretching of H-bonded water), and less intensely at 1630 cm^{-1}. In the case of hydroxyl species, they can be divided in two groups. One group includes OH groups not involved or weakly involved in hydrogen bonding, whose vibrational modes can be found in the 3800-3650 cm^{-1} region, and includesOH terminal groups and also OH isolated groups. The second group includes H-bonded OH groups that contribute to the spectra in the region around 3650-3200 cm^{-1}. This band had several contributions from the water and PVA present in the sol-gel solution.

When using TEOS as silica precursor, a peak around 3740 cm^{-1}, attributed to isolated silanol groups, is sometimes found. In the same region, a band around 3700-3600 cm^{-1} corresponds to vibrational modes of terminal silanols (Figure 3.8). None of these frequencies appear in the FTIR spectra obtained in this work. Silanol groups can be considered intermediates in the route to siloxane networks (figure 3.9), and the absence of the bands referred above indicates that the polymerization reaction was effective and yielded water insoluble, sol-gel-based fiber membrane.

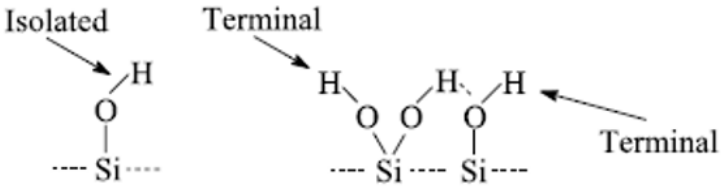


Figure 3.8: Schematic of isolated and terminal hydroxyl groups on silica structures. [102]

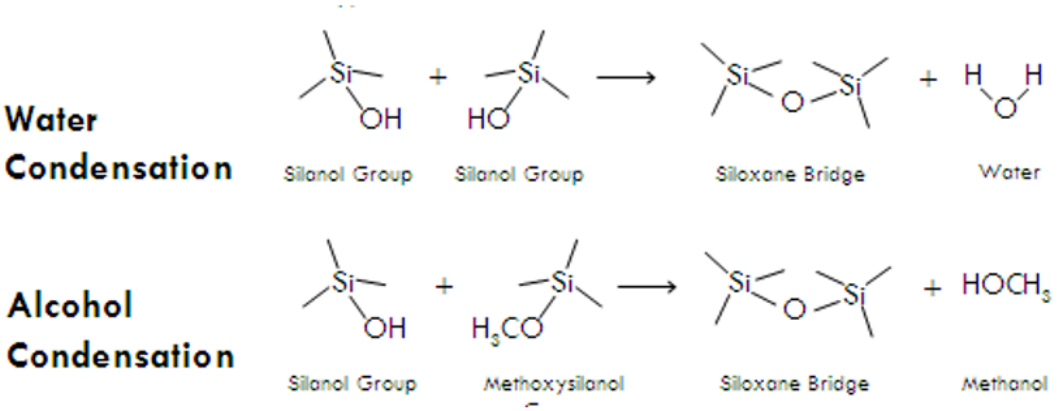


Figure 3.9: The last two stages of the sol-gel process. [103]

The contributions from H- bonded OH functional groups of ethanol are expected in the region between 3300 and 3600 cm^{-1}. Around 880 cm^{-1}, a peak of higher intensity is also expected, due to the contribution of methyl and ethyl vibrations from the alcohol. None of these peaks appear in the spectra obtained, which suggests that the alcohol evaporated during the heating of the sol-gel solution, or electrospinning. When the distance from the needle to the collector is optimum, solvents evaporate before deposition of the fiber on the aluminum foil occurs. The FTIR spectra also reflect the presence of PVA. In Figure3.7, the FTIR spectra of two membranes are compared. The higher intensity of the peaks obtained when using a lower amount of HCl indicates that polymerization was less extensive in this case.

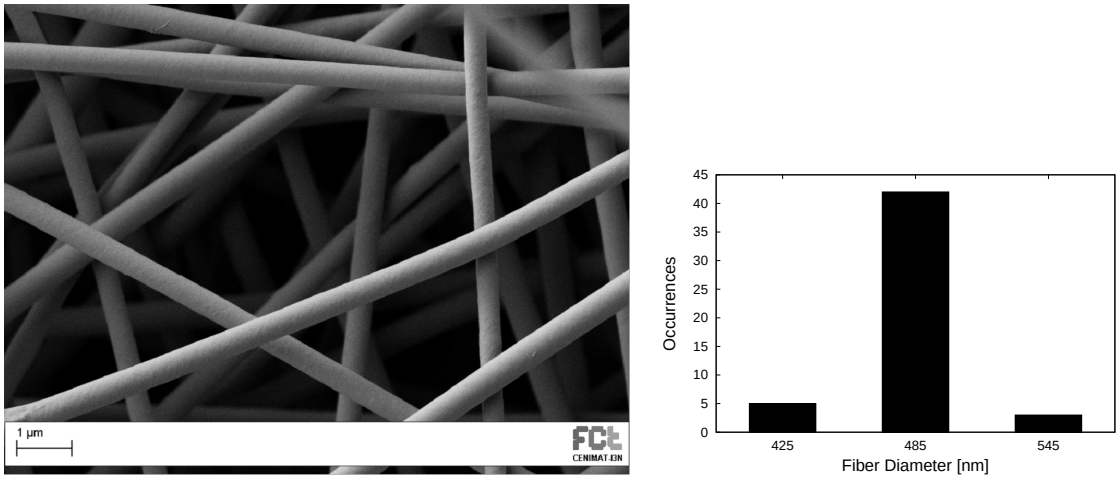
3.7.2 Scanning electron microscopy (SEM)

Although the quality of the fibers obtained was not always excellent (e.g. presence of beads, changes in shape, few fibers per area) through SEM it was possible to determine fiber diameter and determine if the conditions of electrospinning were adequate for producing homogenous fibers. Whenever possible, SEM images are accompanied by a Figure with diameters of fibers.These Figures were produced by counting a population of fifty fibers (independent events) using a Java-based image processing program, namely *ImageJ*. Statical analysis was used for the determination of diameters in each SEM image presented. The fiber diameter distribution (x axis) was obtained by measuring the width of 50 randomly selected data points (y axis) and these diameters were grouped in similar values and displayed in a graph drawn by *Plot*.

3.7.2.1 TMOS Fibers

SEM images were obtained for TMOS-based fibers to assess the influence of each modification in molar ratios on fibers morphology, while trying to maintain electrospinning conditions whenever possible.

In Figure 3.10a, the best fibers obtained are shown. As can be seen, the fibers do not have any beads, have a uniform rounded shape, and a high density of fibers was obtained. The average diameter is found to be 485 nm (Figure 3.10b). Fibers are mostly randomly oriented with narrow fiber diameter distribution.3.10b



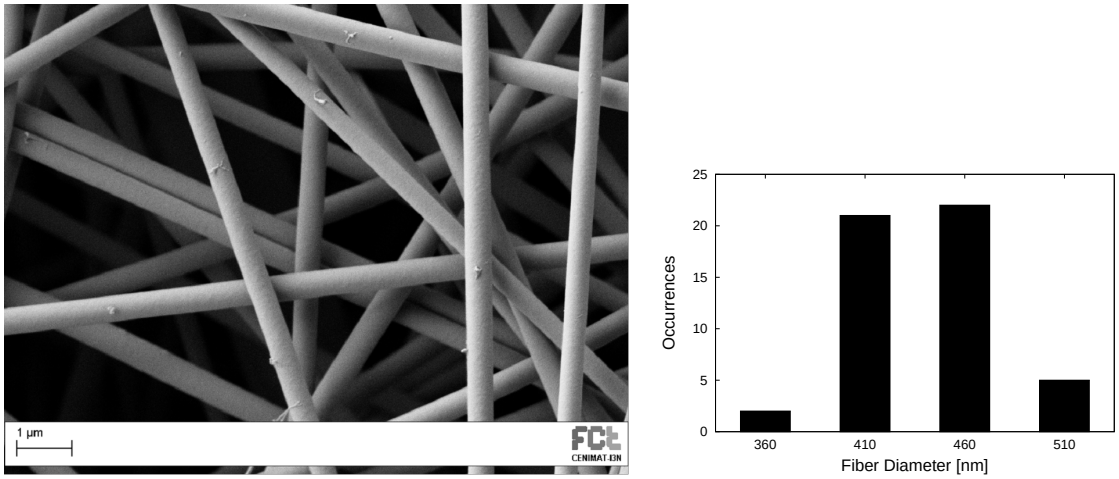
(a) SEM of fibers produced

(b) Distribution of fiber diameters

Figure 3.10: SEM of TMOS-based fibers obtained from solution 1 in (Table 2.5)

Small changes in sol-gel solutions, even when the electrospinning conditions are similar, can lead to differences in the fibers produced, as can be inferred by looking at Figures 3.10b and 3.11b. Changes in the molar ratios of water had a strong influence, though the viscosity of the solutions seemed similar. Fibers obtained from solutions with less water 3.10b had a more uniform range of diameters than fibers obtained from solutions with more water 3.11b

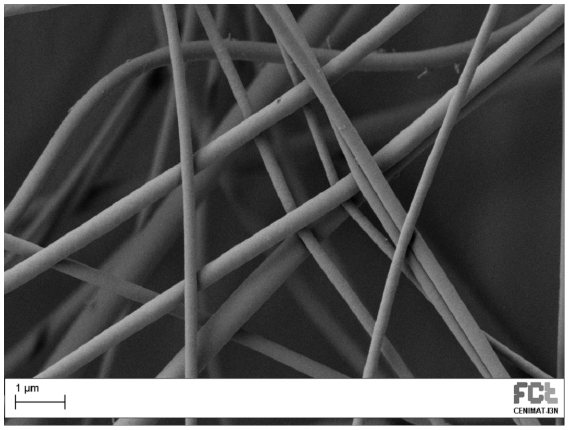
The average diameter is found to be 460 nm 3.10b. This can be explained by interactions between hydroxyl groups from water and methanol that affect the extent of siloxane bond formation. This results in lower rates of condensation (Figure 3.9), lower solution viscosity and consequently wider time window before solidification. This could be confirmed by rheological measurements.



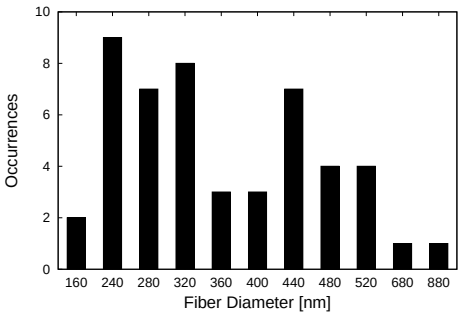
(a) SEM of fibers produced (b) Distribution of fiber diameters

Figure 3.11: SEM of TMOS-based fibers obtained from solution 2 in (Table 2.5)

Similar amounts of precursors can lead to changes in fiber conformation when the aging of the sol-gel solution varies. This aging produces changes of viscosity and thereafter a large range of fiber diameters and fewer number of fibers per area owing to the solidification of solution. These aspects can be observed in Figures 3.11 a) and 3.12 a). Fibers obtained in Figure 3.16a with the sol-gel formulations of Table 2.5 (solution 4) had few beads and a large range of diameters. Since after the addition of PVA the sol-gel solution was not kept at high temperature, but was allowed to cool down, these findings suggests weak links between PVA and the silica precursors, in agreement with the fibers being soluble in water. The other feature of these fibers is that they are similar to PVA fibers (Figure 3.22), having been prepared in a similar way. Fibers with large amounts of PVA have a different texture (Figure 3.16a and Figure 3.16b). These fibers have a narrow distribution, but have very small diameters.The rough aspect of the fibers shown was caused by the beginning of solidification.

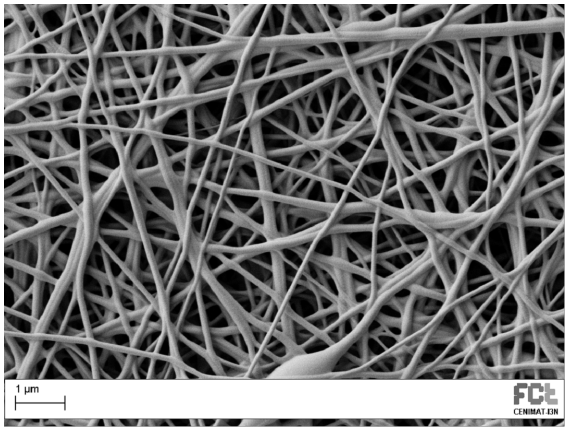


(a) SEM of fibers produced

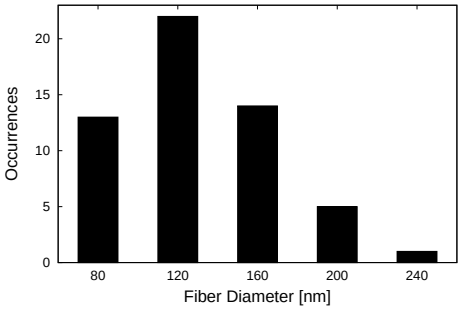


(b) Distribution of fiber diameters

Figure 3.12: SEM of TMOS-based fibers obtained from solution 3 in (Table 2.5)

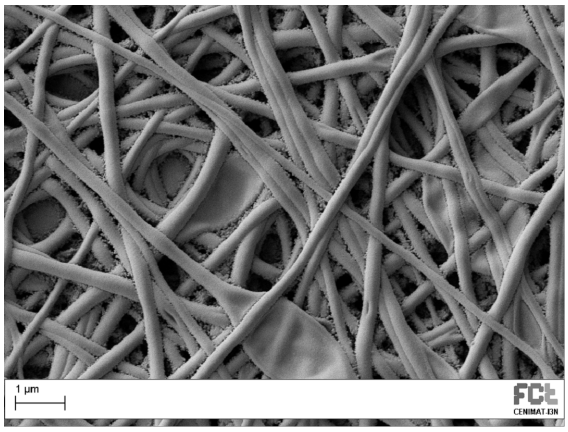


(a) SEM of fibers produced

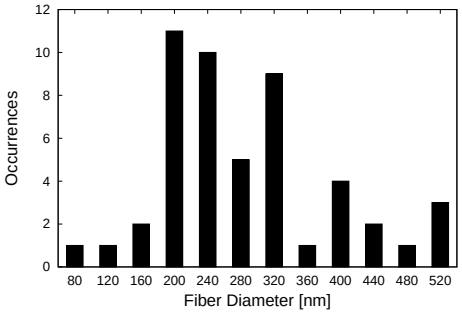


(b) Distribution of fiber diameters

Figure 3.13: SEM of TMOS-based fibers obtained from solution 4 in (Table 2.5)



(a) SEM of fibers produced



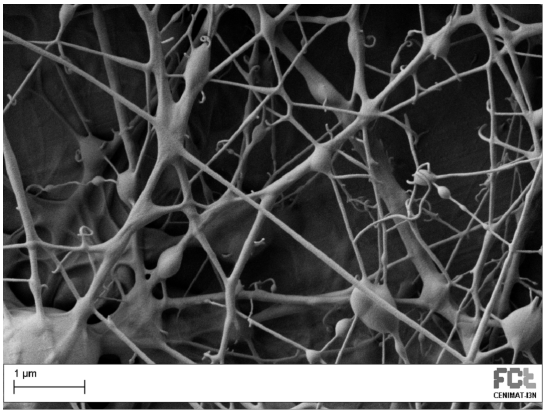
(b) Distribution of fiber diameters

Figure 3.14: SEM of TMOS-based fibers obtained from solution 5 in (Table 2.5)

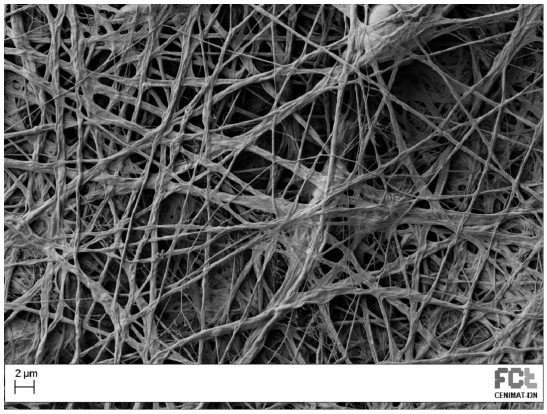
3.7.2.2 TEOS Fibers from bioglass protocol

The optimization of the protocol for silica bioglass was made with different amounts of water and citric acid. Too much water hinders the sol-gel process (Equation (1.2)) [7]. It can also lead to phase separation, as already mentioned. Figure 3.14a shows irregular-shaped fibers with numerous beads, which is consistent with excess water in the sol-gel solution. This suggests that the collector was not far enough to evaporate the solvents from the jet of electrospinning solution.

(a) SEM of TEOS Fibers from solution V.a) (Table 2.7)



(b) SEM of TEOS Fibers from solution V.b) (Table 2.7)



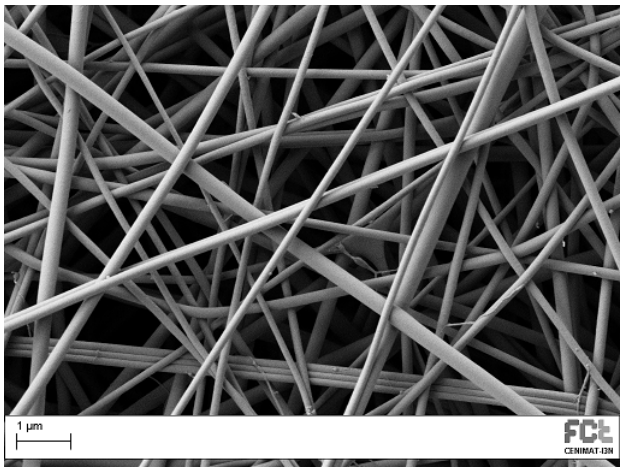
(c) SEM of TEOS Fibers from solution V.c) (Table 2.7)

Figure 3.15: SEM of TMOS-based fibers obtained with the bioglass protocol (Table 2.7)

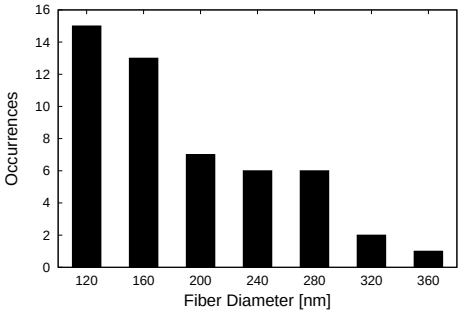
The fibers shown in Figure 3.15c have better quality than those in Figures 3.15a and 3.15b due to the higher amounts of PVA. It is the same case with the fibers in Figure 3.15c. These fibers are similar to those made from PVA 3.22a) and Figure 3.22b), which attests to the contribution of PVA. Consistent with this fact, the fibers shown in figures 3.15a-3.15c were all water soluble.

3.7.2.3 TEOS Fibers

SEM images were obtained for TEOS-based fibers to assess the influence of experimental parameters on fiber morphology.



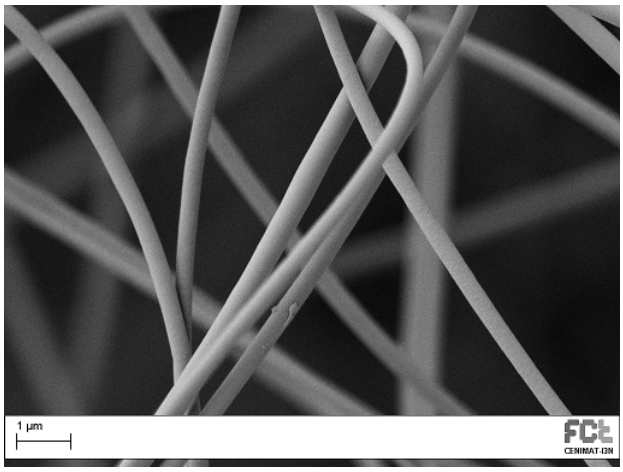
(a) SEM of fibers produced (Table 2.8)



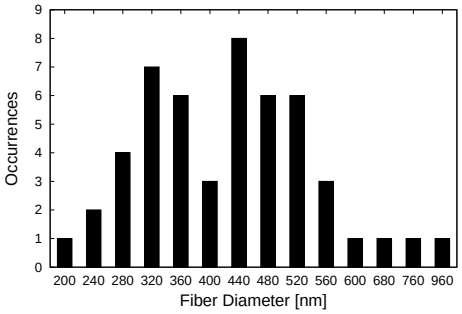
(b) Distribution of fiber diameters

Figure 3.16: SEM of TMOS-based fibers obtained from solution 1 in (Table 2.8)

The fibers shown in Figure 3.16 do not have beads, but on the other hand have a range of diameters between (120nm-360nm), which is wider than desired.



(a) SEM of fibers produced



(b) Distribution of fiber diameters.

Figure 3.17: SEM of TEOS-based fibers obtained from solution 2 in (Table 2.8)

When comparing Figures 3.16 and 3.17, it is possible to notice an improvement in the quality of the fibers. This was achieved by doubling the amount of HCl. This promoted an increase in the efficiency of the sol-gel process, as evidenced by the FTIR spectra in Figure 3.7 (decrease in OH band from water in the 3300-3500 cm^{-1})range.

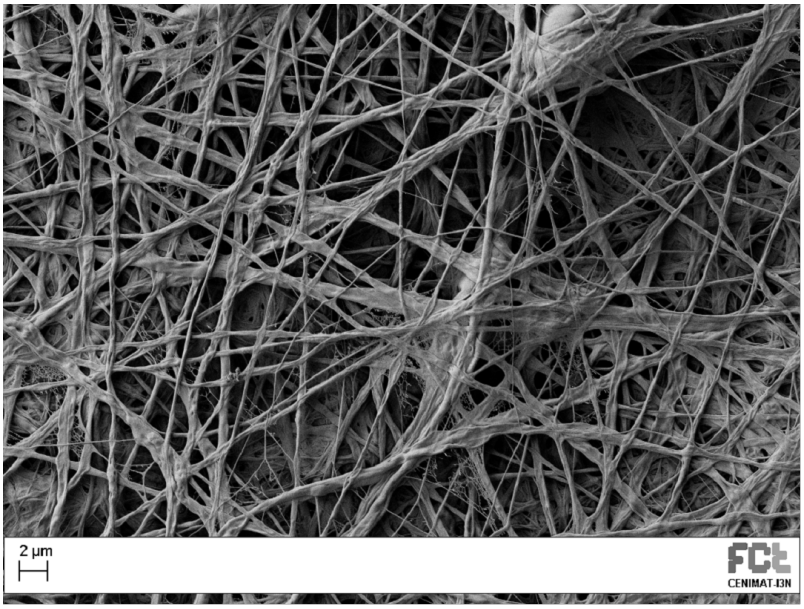


Figure 3.18: SEM of TEOS-based fibers obtained from solution 3 (Table 2.8)

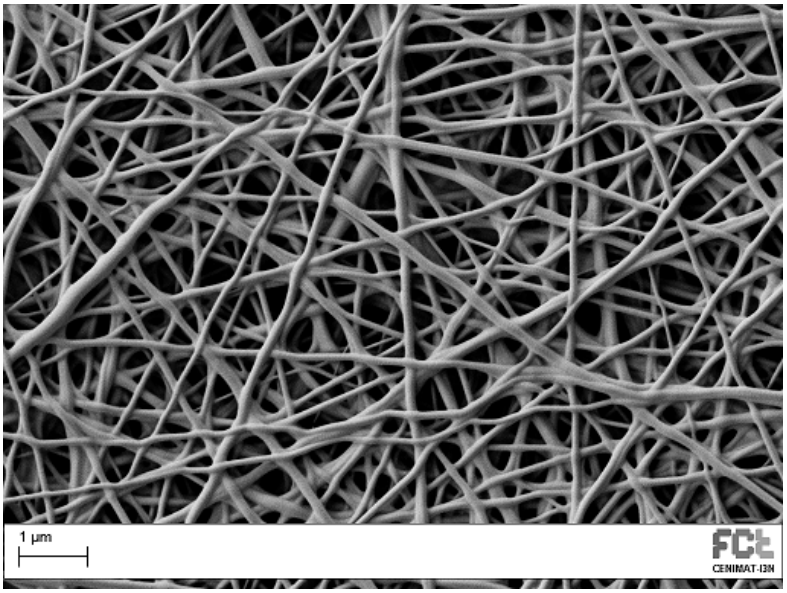
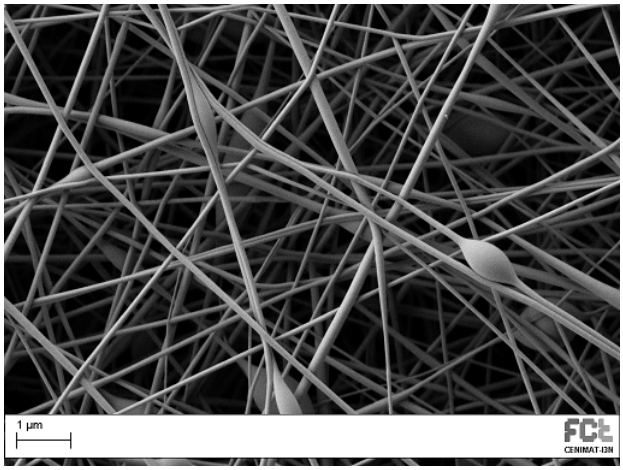
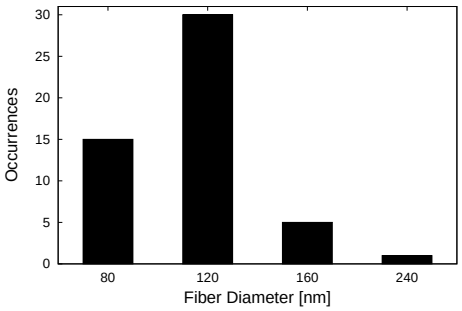


Figure 3.19: SEM of TEOS-based fibers obtained from solution 4 (Table 2.8)

The images of SEM shown in Figures 3.18 and 3.19 are for fibers obtained from sol-gel solutions within different amounts of PVA. For larger amounts of PVA (Figure 3.19) account fibers have more similar diameters and show no beads. Since after the addition of PVA the sol-gel solution was not kept at high temperature, but was allowed to cool down, these findings suggests weak links between PVA and the silica precursors, in agreement with the fibers being soluble in water.

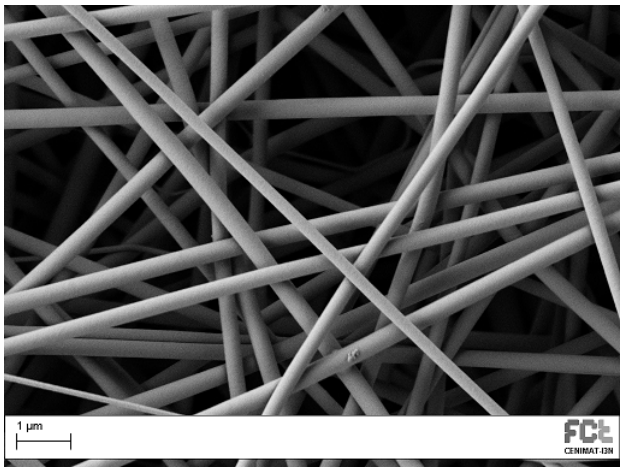


(a) SEM of fibers produced.

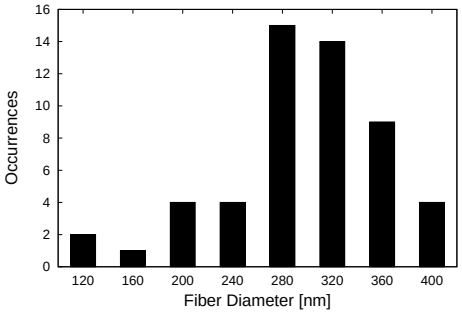


(b) Distribution of fiber diameters.

Figure 3.20: SEM of TEOS-based fibers obtained from solution 5 (Table 2.8)



(a) SEM of fibers produced.

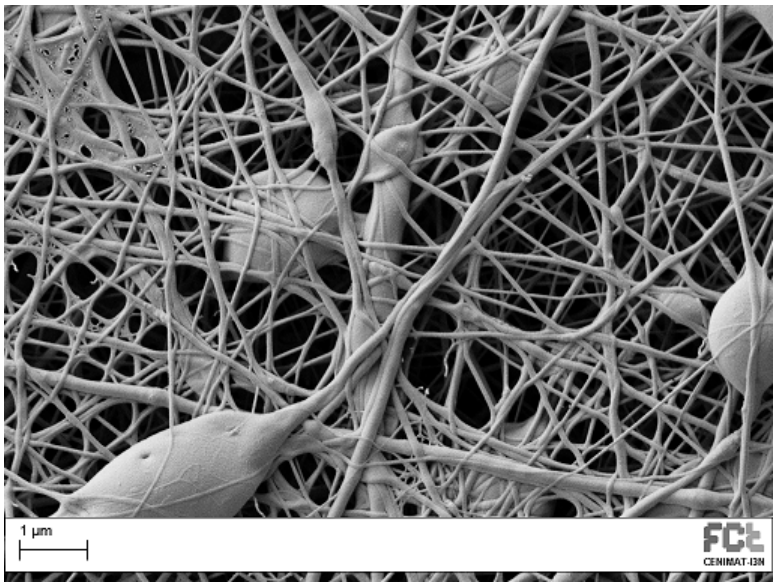


(b) Distribution of fiber diameters.

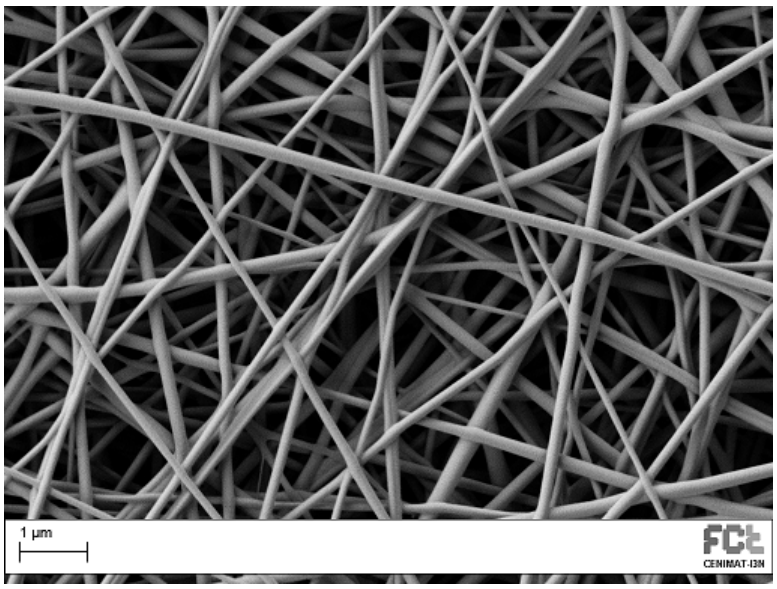
Figure 3.21: SEM of TEOS-based fibers obtained from solution 6 (Table 2.8)

3.7.2.4 Fibers of PVA

With higher amounts of PVA, solutions have higher viscosity and the fibers formed have better quality (Figure 3.22 (b)). These fibers are water soluble and have a fast dissolution.



(a) Fibers obtained using an 18% PVA solution

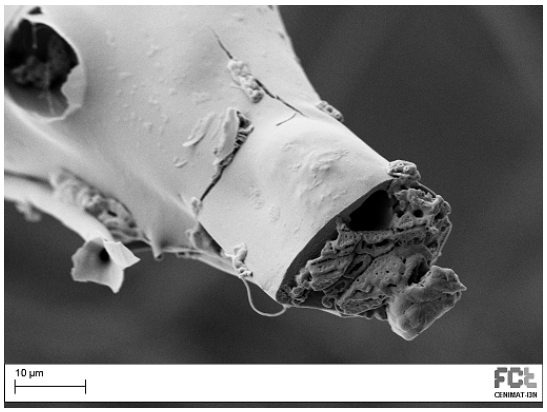


(b) Fibers obtained using an 25% PVA solution

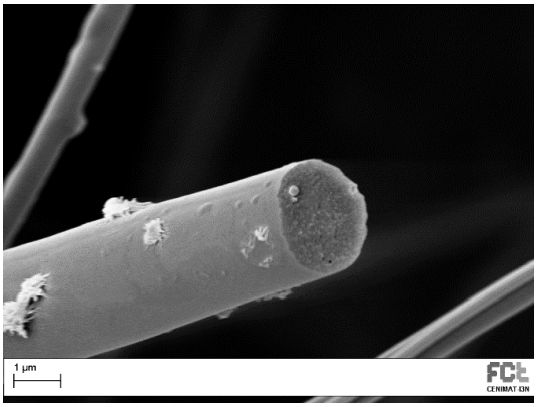
Figure 3.22: SEM of PVA fibers obtained from section 2.3.3

3.7.2.5 Coaxial fibers

To fulfill the goal of this thesis, coaxial fibers were prepared.



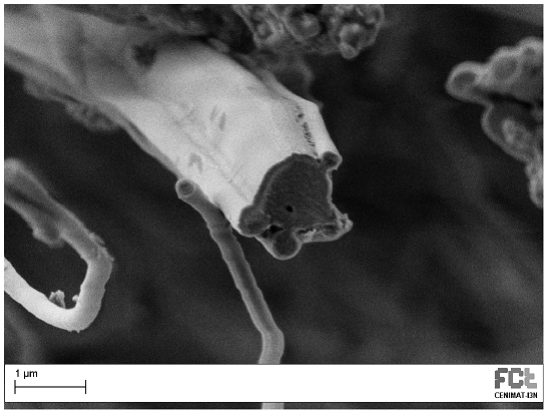
(a) Coaxial fibers obtained from mixture A) from section 2.4



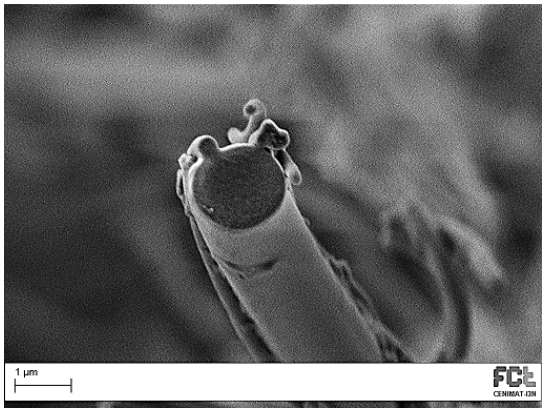
(b) Coaxial fibers obtained from mixture A) from section 2.4

Figure 3.23: SEM of silica-shell/Ion jelly-core coaxial fibers obtained from mixture A) (section 2.4)

The two images (Figure 3.23), showing cross-sections of the fibers, evidence the existence of two regions of different texture (composition), namely the fiber core, exhibiting roughness, and the fiber shell, exhibiting the smooth appearance of silica.



(a)) Coaxial fibers obtained from mixture B) from section 2.4



(b)) Coaxial fibers obtained from mixture B) from section 2.4

Figure 3.24: SEM of silica-shell/Ion jelly-core coaxial fibers obtained from mixture B) (section 2.4).

3.7.2.6 Energy Dispersive Spectroscopy (EDS)

EDS analysis allows the quantification of the chemical elements that it detects. This analysis was made for both coaxial fiber membranes obtained. A) The coaxial fiber membrane A had a shell made of silica sol-gel with PVA, and a core made of Ion jelly with choline DHP.

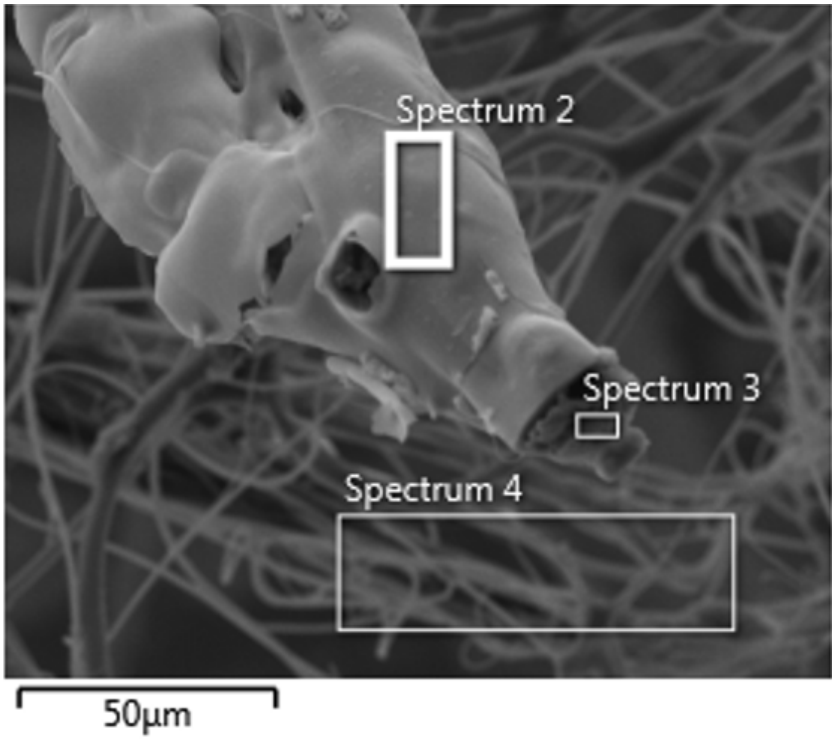
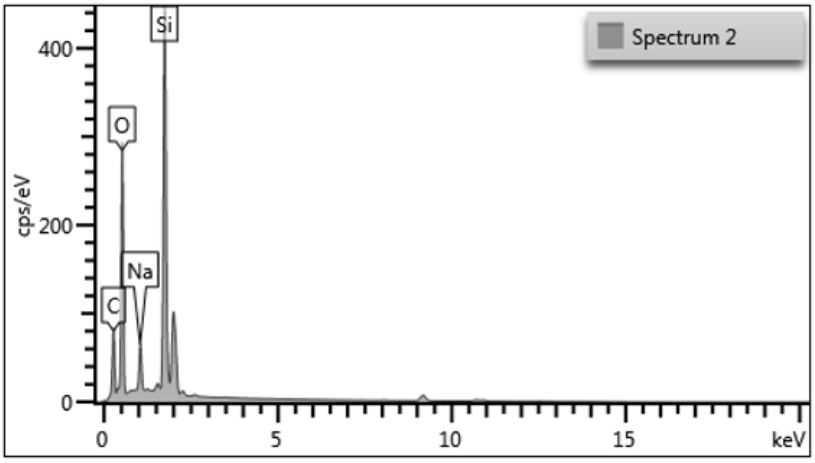


Figure 3.25: Coaxial fiber membrane A) and three sections analyzed in EDS.

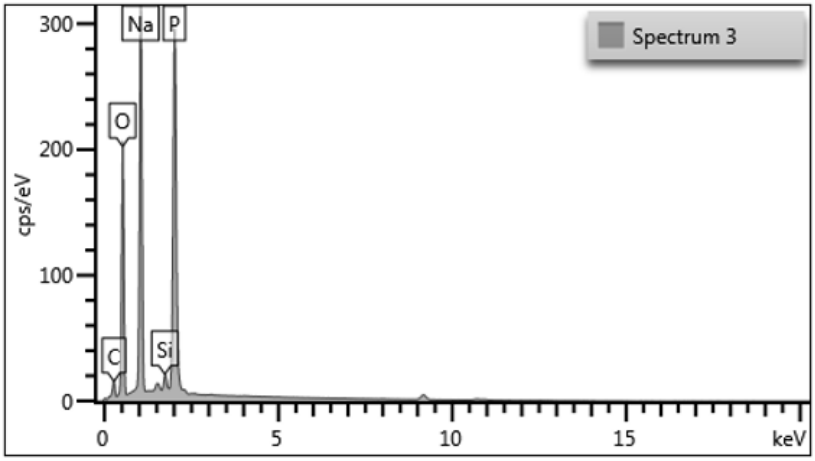
Section 2 is on the shell of the fibers in Figure 3.25(a). The predominant chemical compound detected is silica, as expected. The second major component is oxygen, possibly from TMOS, water or methanol.

Section 3 present in Figure 3.25(b) is on the core of the fibers. The predominant chemical components are phosphorous, which exists in the IL and in the aqueous buffer used, and sodium also present in the latter. The residual presence of silica means good encapsulation of the Ion jelly core.

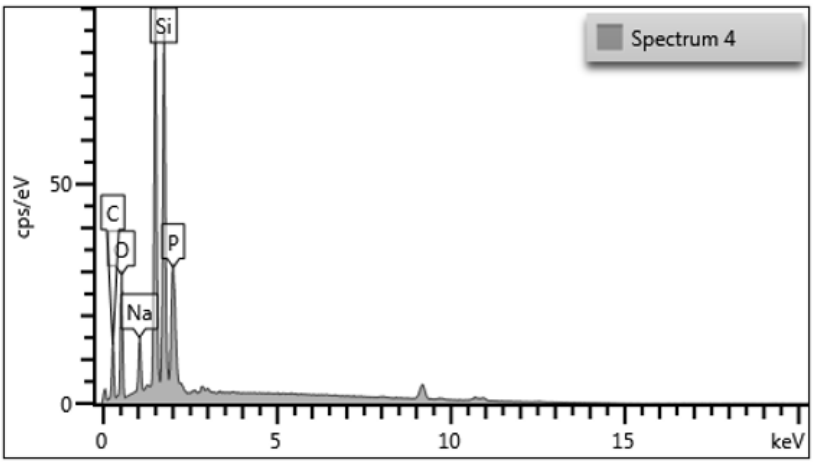
Section 4 on Figure 3.26 is at the shell of the fibers, and again the amount of silica goes up, as for section 2, and the amount of phosphorous goes down. These differences in the chemical nature of shell and core allow to conclude that indeed coaxial fibers were obtained.



(a) EDS analysis of fiber shell



(b) EDS analysis of fiber core



(c) EDS analysis of outer section of fiber membrane.

Figure 3.26: EDS analysis of the membrane in Figure 3.25.

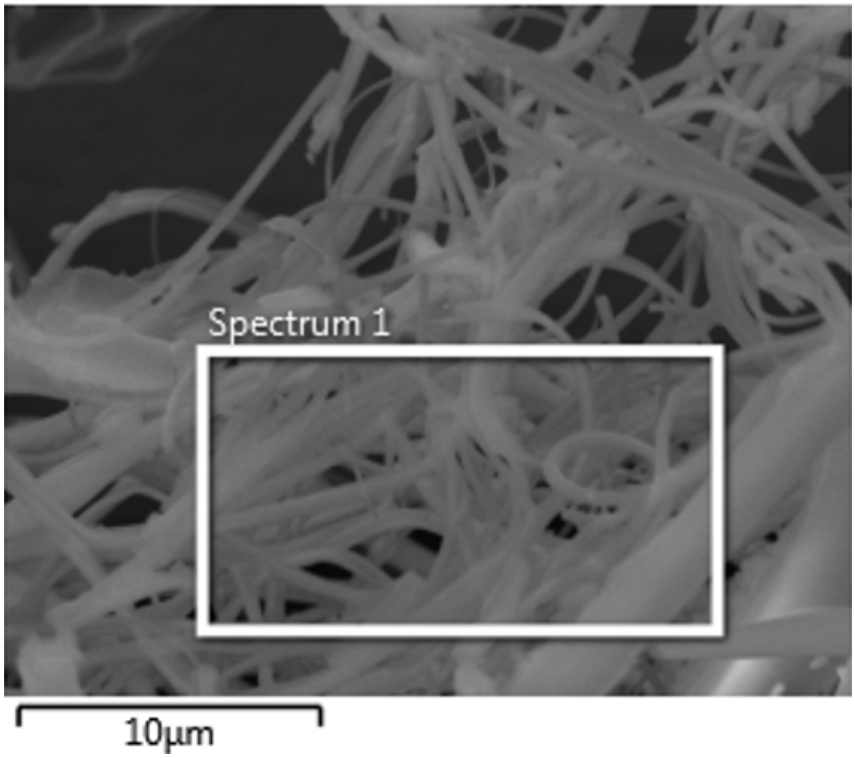


Figure 3.27: Coaxial fiber membrane B) and the section analysed by EDS

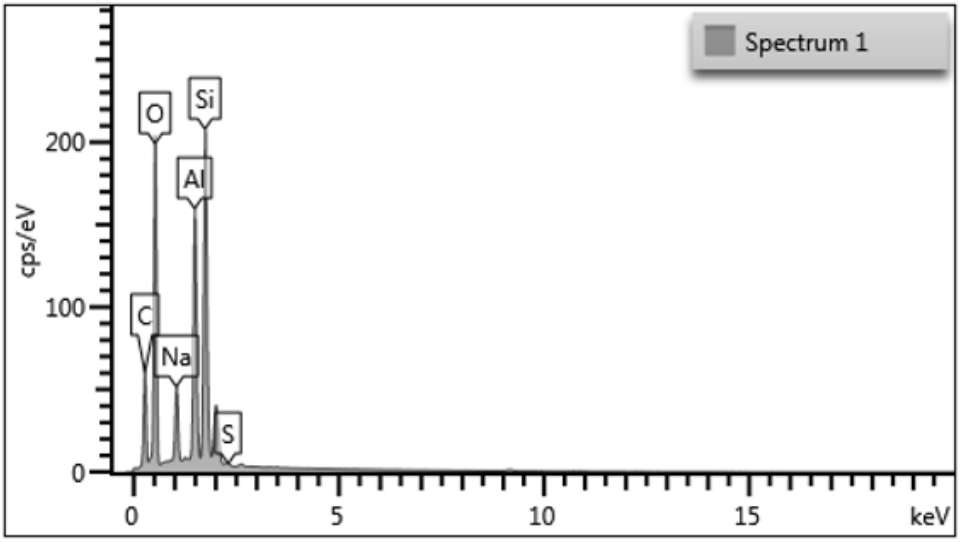


Figure 3.28: EDS analysis of the membrane of Figure 3.27

The spectrum shown in Figure 3.28 does not allow discrimination between fiber shell and core. This does not mean that the encapsulation was not effective. The IL interacts with photons from SEM and this makes it difficult to analyze fibers. In spite of the contrast revealed in SEM images, the radiation does not penetrate dep enough to allow the chemical characterization of the fibers.

3.7.3 Assessment of fiber membrane solubility

Water solubility is an important characteristic to study because one possible utilization of the fiber membranes is as functional materials for bioremediation in aqueous media. The sol-gel process through acid catalysis produces al water insoluble silica matrix, as wanted. However, the sol-gel solution does not have the viscosity necessary for electrospinning. This was overcome by addition of aqueous solution of PVA, which is one of the polymers more widely used and therefore produced in huge amounts. [104] But PVA is easily dissolved in water. The main reason to choose this polymer was the flexibility and the porosity that it lends to sol-gel fibers. These characteristics vary according to the molecular weight of PVA (degree of polymerization-size of polymer) and percentage of hydrolysis (ratio of hydrophilic alcohol group to hydrophobic acetate group). The molecular weight and percentage of hydrolysis of PVA are inversely proportional to water solubility. [104] The biggest concern in blending PVA with the sol-gel silica solution was the increase in viscosity that it caused. This made the solution solidify faster and less electrospinnable, and induced water solubility of the resulting membrane. To test these effects, the amount of PVA used was varied.3.7

Table 3.7: Different molar ratios used to prepare fiber membranes and respective water solubility

Membrane (compounds-molar ratios)	**Water solubility**
TEOS:H_2O:ethanol:citric acid:PVA $1.00 : 91.33 : 17.26 : 51.07 : 2.79 \times 10^{-3}$	Yes, instantly
TEOS:H_2O:ethanol:citric acid:PVA $1.00 : 91.33 : 17.26 : 51.07 : 4.48 \times 10^{-3}$	Yes, instantly
TEOS:H_2O:ethanol:citric acid:PVA $1.00 : 91.33 : 17.26 : 51.07 : 5.64 \times 10^{-3}$	Yes, instantly
TEOS:ethanol:H_2O:HCl:PVA $1.00 : 2.10 : 9.00 \times 10^{-1} : 1.40 \times 10 - 4 : 5.29 \times 10^{-1}$	No
TEOS:ethanol:H_2O:HCl:PVA $1.00 : 2.11 : 9.00 \times 10^{-1} : 2.80 \times 10 - 4 : 5.29 \times 10^{-1}$	No
TEOS:ethanol:H_2O:HCl:PVA $1.00 : 26.03 : 3.44 : 2.80 \times 10^{-4} : 1.40 \times 10^{-4}$	No
TEOS:ethanol:H_2O:HCl:PVA $1.00 : 26.40 : 4.24 : 2.80 \times 10^{-4} : 5.29 \times 10^{-1}$	No
TEOS:ethanol:H_2O:HCl:PVA $1.00 : 2.10 : 9.00 \times 10^{-1} : 3.31 \times 10 - 7 : 6.17 \times 10^{-4}$	No
TEOS:ethanol:H_2O:HCl:PVA $1.00 : 2.10 : 9.00 \times 10^{-1} : 3.31 \times 10 - 7 : 4.32 \times 10^{-4}$	No
TMOS:Methanol:H_2O:HCl:PVA $1.00 : 2.01 : 1.09 : 7.85 \times 10^{-1} : 3.45 \times 10^{-4}$	No
TMOS:Methanol:H_2O:HCl:PVA $1.00 : 17.47 : 4.05 : 4.13 \times 10^{-2} : 3.45 \times 10^{-4}$	No
TMOS:Methanol:H_2O:HCl:PVA $1.00 : 17.22 : 3.29 : 4.13 \times 10^{-2} : 3.45 \times 10^{-4}$	No
TMOS:H2O:HCl:PVA $1.00 : 2.80 : 4.33 \times 10^{-4} : 3.72 \times 10^{-5}$	Yes
TMOS:H2O:HCl:PVA $1.00 : 2.80 : 4.33 \times 10^{43} : 1.05 \times 10^{-4}$	Yes

Membranes with higher amounts of PVA or inadequate heating time had a similar aspect on SEM as PVA membranes. Membranes that dissolve in water can be produce and adapted for different purposes. For instance, they may be used as scaffolds for impregnating active ingredients in biomedical applications [64] , drug delivery [105] , sustained release, implant application [106] and enzyme [28] and protein separation. In addition to applications in the health area, they can also be used for environmental applications, such as acid recovery , alcohol dehydratation, and desalination of dye solutions. After 5 day immersion in water, the membranes that did not immediately dissolve in water remained intact 3.29.

Figure 3.29: Picture of membrane of TMOS prepared from solution 1 (Table 2.5)

It was also necessary to study how long PVA should be allowed to interact with the silica precursor for lower water solubility while ensuring sufficient time for electrospinning. It was easy to cross the line in this respect, because when an imperceptible part of the solution solidified, it triggered gelation and the whole system became solid in seconds. When the solution with added PVA was heated for 1 h, the resulting membrane dissolved in part (around 20%), and exhibited high transparency when wet. When it was taken out of the aqueous environment, the membrane curled up and tended to break with the water weight. When the solution with PVA was heated for 1 h 15 min, the membrane obtained dissolved in water in a lower amount (around 10%), and when removed from water, was more easily straightened up on a glass plate. The propensity to curl was less than with the previous membrane. The best results were obtained when the sol-gel solution containing PVA was heated for 1 h 30 min. Visually, the membranes obtained in this manner did not dissolve in water. Also these membranes remained white when immersed in water, as opposed to translucent. And they did not curl up.

3.7.4 Conductivity measurements

In order to assess the influence of IL in films, films were prepared with different amounts of IL and gelatin, as shown in table 3.8.

Table 3.8: Ion jelly films with choline DHP

Mixtures	Concentration	Conductivity (S cm^{-1})
0	8% Gelatin	2.083×10^{-9}
1	8% Gelatin + 8% IL	2.083×10^{-5}
2	10% Gelatin + 10% IL	4.167×10^{-5}
3	31% Gelatin + 18% IL	7.000×10^{-3}
4	44% Gelatin + 17% IL	1.225×10^{-4}
5	32% Gelatin + 17% IL	1.575×10^{-4}

Film identified as 0 (Table 3.8) was prepared without IL, for comparison in order to perceive the influence of the IL. In comparison with values in the literature [63] the films with IL have conductivity values between $10^{-4} and 8 \times 10^{-2}$ S cm^{-1}). The ionic enviroment is good for enzyme immobilization because it allows ion exchange and promotes the reduction and oxidation of compounds used by oxido-reductases.

3.7.4.1 TMOS-based membranes

The conductivity of a sol-gel membrane was measured in order to understand if the cotton-like structures obtained were caused by membrane conductivity. The result obtained was 3.467×10^{-9} S cm^{-1}. This value is low for electrically conductive membrane. Nevertheless, the limits of the equipment may have been exceeded.

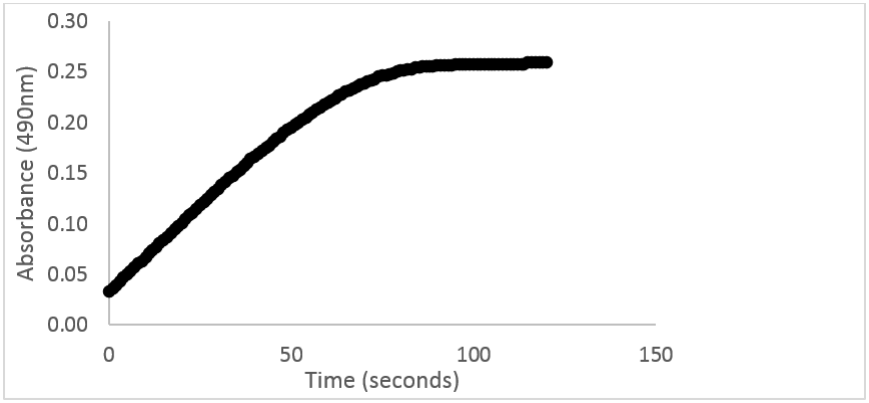
3.8 Enzyme activity measurements

Figure 3.30a shows a typical plot of absorbance vs. time obtained when assaying the activity of HRP in aqueous buffer. As shown in Figure 3.30b, the increase in absorbance was linear for about 60 s, which allowed the calculation of initial rates, taken as a measure of enzyme activity. The slope of the curve levels off as the substrate is depleted.

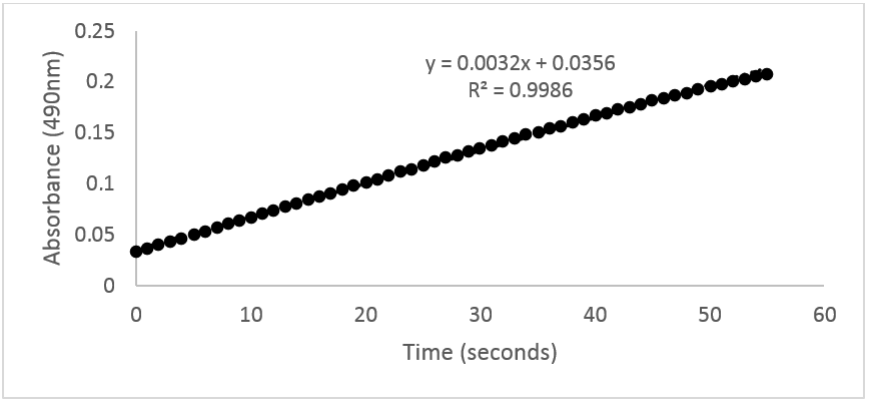
Table 3.9: Enzyme activity assays in different media

Corresponding sections with previous chapter	Essay	Velocity of enzyme ($nmols^{-1}mg^{-1}$)
3.8.1	Phosphate buffer, pH 7.0	9.9
3.8.2	Phosphate buffer, pH 6.0	21.0
3.8.3	Phosphate buffer, pH 7.0, with choline IL	3.1
3.8.3	Phosphate buffer, pH 7.0, with 3 times more choline IL	0.62
3.8.4.1	Acetate buffer ∼17	0.93
3.8.4.2	Acetate buffer ∼0.3 M	8.0

In several assays, phosphate buffer was used at pH 7.0, based on an article [36] whose authors studied the behavior of free and immobilized HRP and found that the enzyme performed better at pH 7.0, while the free enzyme performed better at pH 6.0. In our case, the specific activity of the enzyme practically doubled when the pH changed from 7.0 to 6.0.



(a) Enzyme activity assay for HRP in phosphate buffer pH=7



(b) Initial rate period from previous graph

Figure 3.30: Enzyme activity assay

Choline DHP is referred in the literature as providing a protective environment for the immobilization of proteins, enzymes and other biological structures [58]. However, in our work the concentration of this IL used, which is within the range referred in the literature, led to initial rates that were about 3 times lower than in the absence of IL. This effect became more pronounced when the amount of choline DHP was increased 3 times, which lowered enzyme activity about 15 times when compared to buffer alone. This may be caused by a negative effect of the IL on the enzyme. In this case, the enzyme is in a medium with a lot of water and can easily adapt to the presence of the IL. The resulting conformation may lead to lower enzyme activity. If the medium had less water, the enzyme might be more rigid and less able to adapt to the solvent, thereby preserving its activity.

To overcome the difficulties with early gelation of Ion jelly solutions, an acetate buffer (pH = 4.5) was prepared with the minimum amount of acetic acid that was found to prevent solidification. When the enzyme was added to this solution, its activity was about 11 times lower in comparison with the phosphate buffer. This must be due to the high ionic strength imparted by the high concentration of both acetic acid (≈17M) and acetate. Given that pH 4.5 was reported to cause only around 15% loss of enzyme activity compared to pH 6.0 [36], other experiments were performed to study the influence of ionic strength on enzyme activity.

The acetate buffer was thus diluted about 50 times, to yield a ≈0.3M concentration. In this case, the assay was performed similar with the previously with an acetate buffer 50 times less concentrate. As seen in the table, the enzyme worked well in this buffer, with enzymatic rates similar to those in phosphate buffer at pH 7.0.

To assess the influence of encapsulation of HRP in silica fibers, a typical sol-gel solution (solution 1, Table 2.5) was prepared, and enzyme was added. No pH adjustment was made before electrospinning. The fiber membrane thus prepared did not exhibit enzyme activity. The enzyme was in contact with the silica medium at low pH for around 45 min before gelation took place, which may have led to denaturation. That the enzyme was irreversibly inactivated was confirmed by adding phosphate buffer at pH 6.0 and performing a standard activity test, which resulted negative. No pink color was detected, even after 14 h at room temperature, or after leaving the preparation overnight at 35 °C. In this case, to make sure the medium that remained on the membrane still had the required reagents to perform the assay, a small amount of enzyme solution in phosphate buffer was added, and in seconds the medium became pink, which intensified with time.

Chapter 4

Conclusions and Future Work

4.1 Conclusions

The development of biocatalytic electrospun membranes, obtained from silica sol-gel solutions, has been reported in several articles. However, most of these reports involve the immobilization of the enzyme on to the fiber membrane, whereas in our work the enzyme was incorporated in the membrane at the time it was electrospun. As for core-shell fiber membranes with entrapped enzyme, prepared from two types of solutions, they are documented in only a few recent articles. And none of these articles report on the use of an Ion jelly based fiber core, as was the goal of this work.

One major advantage of producing fibers using the silica sol-gel process is that it is possible to obtain fibers that are insoluble in water, which may allow their use for environmental applications, such as bioremediation of aqueous streams.

Many silica sol-gel solutions were prepared to study the influence of each compound, or combination of compounds present, their amounts, electrospinning conditions, and many other factors that affect the gelation of the solution. Fast solidification was always a great concern, together with the issue of effective cleaning of the electrospinning needles.

Through several modifications to available protocols, it was possible to conclude that silica precursor/water molar ratios of 1:2 were a good approach. The impact of temperature, not only at which the sol-gel solution was initially prepared, but at which the solution was kept after addition of PBA, allowing the cross-linking of silica and PVA, was found to be very important, 60 °C being the optimum temperature. Lower temperature resulted in water soluble membranes. The time window for making the fibers was very dependent on the conditions used in preparing the sol-gel mixture with added PVA. The full solidification of the sol-gel solution occurred within seconds of the first signs of polymeric agglomeration.

To look for conditions that might be adequate for immobilizing HRP in Ion jelly fiber cores, catalytic activity assays were performed with horseradish peroxidase. Experiments

with choline DHP, an IL known to support the activity and stability of biological material, showed that the amount of IL is important, as evidenced by the 15 times decrease in HRP activity when the amount of choline DHP was increased 3 times.

The processing of solutions of gelatin with IL was very difficult, and it was only possible to achieve adequate conditions for electrospinning by heating the syringe directly. But such a procedure is hard to control, and there was always the danger of enzyme denaturation. To overcome this problem, acetic acid was added to extend the time before gelation. This created an environment that proved harmful to the enzyme, even when the pH was increased by addition of acetate and creation of a buffer at pH 4.5, a pH at which HRP is known to express a large proportion of its optimum catalytic activity. However, the ionic strength of the buffer thus prepared was extremely high, and led to a considerable loss of enzyme activity. The negative effect of high ionic strength was confirmed by testing an acetate buffer 50 times less concentrated, in which the enzyme performed quite well. But with the dilution of the acid, the solution was found to suffer gelation around 36 °C.

It was very difficult to optimize the conditions of the electrospinning chamber for producing co-axial fiber membranes with an Ion jelly core, due to the influence of temperature on the solidification of both the sol-gel solution. Minimum temperature had to be 35 °C in the case of gelatin, or 30 °C acetate buffer was used.

In case of gelatin solution minimum of 35°C when prepared with water, or 30 °C when prepared with acetate buffer. For the higher the temperature in the chamber, the more difficult it was to maintain the humidity needed to produce fibers (always above 16%). The other difficulty was to control the drop in the Taylor cone and to project the jet from the drop efficiently onto the collector. This problem is mainly due to the instability of both solutions, taking into account the high tension used (20 kV). The Ion jelly has electrocondutivity, and this led to the production of 3D structures which interfered in the stress field.

Since both of the solutions used in the electrospinning process were prone to create instability in the system, changes in the formulation used for the fiber cores were performed. One of these was the use of a gelatin solution with low concentration (5.5% w/w), without IL, but even at low flow rates (0.09 mL/h) the result was successive drops falling. This decrease in flow rate creates one of two problems: the need to increase the time of electrospinning to incorporate a significant amount of enzyme, which is not feasible due to the solidification of the sol-gel solution (it would have to remain fluid for at least 4 h of electrospinning), or the need to increase the amount of enzyme used, which is expensive. The enzyme activity assays performed with lower amounts of gelatin were negative, quantitatively or visually.

When the Ion jelly solution was replaced with PVA solution, an increase in the stability

of electrospinning conditions was achieved, but the time of collection of the membrane was too short for a sufficient amount of enzyme to be encapsulated, and in fact visual observation assays were negative for enzyme activity.

Co-axial electrospinning is a powerful tool for producing fibers that combine different materials, including biological molecules, which creates new opportunities for applications. Although it proved very challenging to obtain solutions with appropriate viscosity for electrospinning, it was possible to achieve a time window that allowed the manufacture of co-axial, silica-shell, Ion jelly-core fiber membranes.

Although it was not yet possible to obtain biocatalytic core-shell membranes with immobilized HRP that tested positive for enzyme activity, progress was made in that direction, namely by using ammonia for increasing the pH to values between 3.5 and 5.0 At these pH values films were prepared that showed good enzymatic activity. It should be possible to use this approach to meet the goal of this thesis.

4.2 Future Work

I. Simple fibers

   (a) To increase the pH of the sol-gel solution before addition of the enzyme. This can be done by adding an ammonia solution (NaOH solution brings about fast solidification). This approach was followed when preparing films and these tested positive for enzyme activity.

II. Coaxial fibers

   (a) Do more assays with gelatin, trying to find the right amount that ensures fluidity of the solution long enough to spin fibers;

   (b) When using a PVA solution for the fiber cores, electrospinning conditions were more stable than with the gelatin/IL solution. This approach for making fiber cores in not new, but given enough electrospinning time, and a better control of temperature and humidity, should be appropriate for making fibers cores within which the enzyme can be immobilized;

   (c) Test other compounds for making fiber cores, such as cellulose acetate, or polycaprolactone, which are biocompatible polymers, similarly to PVA.

Bibliography

[1] L. A. Dahili, I. Kelemen-horváth and T. Feczkó, '2 , 4-dichlorophenol removal by purified horseradish peroxidase enzyme and crude extract from horseradish immobilized to nano spray dried ethyl cellulose particles', *Process Biochemistry*, vol. 50, no. 11, pp. 1835–1842, 2015.

[2] X. Ye, L.-Y. Wong, X. Zhou and A. M.Calafat, 'Urinary concentrations of 2,4-dichlorophenol and 2,5-dichlorophenol in the u.s. population (national health and nutrition examination survey, 2003-2010):trends and predictors', *Environmental Health Perspectives*, vol. 122, no. 4, pp. 351–356, 2014.

[3] A. A. Aghapour, G. Moussavi and K. Yaghmaeian, 'Degradation and cod removal of catechol in wastewater using the catalytic ozonation process combined with the cyclic rotating-bed biological reactor', *Journal of Environmental Management*, vol. 157, pp. 262–266, 2015.

[4] H. Adamu, P. Dubey and J. A. Anderson, 'Probing the role of thermally reduced graphene oxide in enhancing performance of tio 2 in photocatalytic phenol removal from aqueous environments', *Chemical Engineering Journal*, vol. 284, pp. 380–388, 2016.

[5] N. Singh and C. Balomajumder, 'Journal of water process engineering simultaneous removal of phenol and cyanide from aqueous solution by adsorption onto surface modified activated carbon prepared from coconut shell', *Journal of Water Process Engineering*, vol. 9, pp. 233–245, 2016.

[6] B. Kumar, V. K. Verma, M. Mishra, J. Tyagi, C. S. Sharma, B Akolkar, B. Kumar, V. K. Verma, M. Mishra and J. Tyagi, 'Human and ecological risk assessment : an international quantification of nitrophenols , chlorophenols , and hexachlorocyclohexanes in agricultural soils in the vicinity of industrial area for the assessment of human health hazard and risk', *Human and Ecological risk Assessment: An International Journal*, vol. 22, no. 1, pp. 39–49, 2016.

[7] M. Chen, P. Xu, G. Zeng, C. Yang, D. Huang and J. Zhang, 'Bioremediation of soils contaminated with polycyclic aromatic hydrocarbons , petroleum , pesticides , chlorophenols and heavy metals by composting : applications , microbes and future research needs', *Biotechnology Advances*, vol. 33, no. 6, pp. 745–755, 2015.

[8] X. Duan, S. C. Corgiø, D. J. Aneshansley, P. Wang, L. P. Walker and E. P. Giannelis, 'Hierarchical hybrid peroxidase catalysts for remediation of phenol wastewater', *ChemPhysChem*, vol. 6900, pp. 974–980, 2014.

[9] S. Roy, I. Das, M. Munjal, L. Karthik, G. Kumar, S. Kumar, K. Venkata and B. Rao, 'Isolation and characterization of tyrosinase produced by marine actinobacteria and its application in the removal of phenol from aqueous environment', *Frontiers in Biology*, vol. 9, no. 4, pp. 306–316, 2014.

[10] J Michałowicz and W Duda, 'Phenols – sources and toxicity', *Polish Journal of Environmental Studies*, vol. 16, no. 3, pp. 347–362, 2007.

[11] L. Barraza, 'A new approach for regulating bisphenol a for the protection of the public 's health', in *Practical approaches to critical challenges*, 2013, pp. 9–12.

[12] L. E. Dodge, P. L. Williams, M. A. Williams, S. A. Missmer and T. L. Toth, 'Paternal urinary concentrations of parabens and other phenols in relation to reproductive outcomes among couples from a fertility clinic', *Environmental Health Perspectives*, vol. 123, no. 7, pp. 665–671, 2015.

[13] M. S. A. Palma, H. Horn, M. Zilli, G. Pigatto and A. Converti, 'A new enzymatic process for the treatment of phenolic pollutants', *Brazilian archives of biology and technolgy*, vol. 56, no. August, pp. 653–662, 2013.

[14] L. L. Bergeson, 'Epa proposes significant new use rule for cartain nonylphenol and nonylphenol ethoxylates', *Environmental Quality Management*, vol. 24, no. 3, pp. 105–109, 2015.

[15] C. Zhong, M. He, H. Liao, B. Chen, C. Wang and B. Hu, 'Polydimethylsiloxane/covalent triazine frameworks coated stir bar sorptive extraction coupled with high performance liquid chromatography-ultraviolet detection for the determination of phenols in environmental water samples', *Journal of Chromatography A*, vol. 1441, pp. 8–15, 2016.

[16] T. Prevc, A. Levart, I. K. Cigié, J. Salobir, N. P. Ulrich and B. Cigié, 'Rapid estimation of tocopherol content in linseed and sunflower oils-reactivity and assay', *Molecules*, vol. 20, no. 8, pp. 14 777–14 790, 2015. DOI: 10.3390/molecules200814777.

[17] E. Durand, J. Lecomte and P. Villeneuve, 'From green chemistry to nature : the versatile role of low transition temperature mixtures r o', *Biochimie*, vol. 120, pp. 119–123, 2016.

[18] N. P. Tarasova, A. S. Makarova and F. I. Ingel, 'Systemic approach to the development of green chemistry', *Pure and Applied Chemistry*, vol. 88, pp. 37–42, 2016.

[19] A. Gałuszka, Z. Migaszewski and J. Namieśnik, 'The 12 principles of green analytical chemistry and the significance mnemonic of green analytical practices', *Trends in Analytical Chemistry*, vol. 50, pp. 78–84, 2013.

[20] T. Coradin, E. Guibal, N. Baccile, F. Babonneau, B. Thomas and T. Coradin, 'Introducing ecodesign in silica sol – gel materials', vol. 19, no. 45, pp. 8497–8692, 2009.

[21] C. Herrmann, M. Hauschild, T. Gutowski and R. Lifset, 'Life cycle engineering and sustainable manufacturing', *Journal of Industrial Ecology*, vol. 18, no. 4, pp. 471–477, 2014.

[22] A. Kunamneni, I. Ghazi, S. Camarero, A. Ballesteros, F. J. Plou and M. Alcalde, 'Decolorization of synthetic dyes by laccase immobilized on epoxy-activated carriers', *Process Biochemistry*, vol. 43, pp. 169–178, 2008.

[23] C. S. Karigar and S. S. Rao, 'Role of microbial enzymes in the bioremediation of pollutants : a review', *Enzyme Research*, vol. 2011, pp. 1–11, 2011.

[24] M Vidali, 'Bioremediation . an overview *', *Pure and Applied Chemistry*, vol. 73, no. 7, pp. 1163–1172, 2001.

[25] S. E. Fantroussi and S. N. Agathos, 'Is bioaugmentation a feasible strategy for pollutant removal and site remediation ?', *Current opinian in microbiology*, vol. 8, pp. 268–275, 2005.

[26] A. Singh, S. Kumari and T. K. Pal, 'In silico analysis for laccase-mediated bioremediation of the emerging pharmaceutical pollutants preparation of ligands', *International Journal Bioautomation*, vol. 19, no. 4, pp. 423–432, 2015.

[27] J. W. Dale, W. B. Ed and J. Wiley, *Understanding Microbes: An introduction to a small world*. Wiley-Blackwell, 2013.

[28] L. Wu, J. Sun, Z. Lv and Y. Chen, 'In-situ preparation of poly (ether imide)/ amino functionalized silica mixed matrix membranes for application in enzyme separation', *Materials and Design*, vol. 92, pp. 610–620, 2016.

[29] S. Ghasempur, S.-F. Torabi, S.-O. Ranaei-Siadat, M. Jalali-Heravi, N. Ghaemi and K. Khajeh, 'Optimization of peroxidase-catalyzed oxidative coupling process for phenol removal from wastewater using response surface methodology', *Environmental science&Technology*, vol. 41, no. 20, pp. 7073–7079, 2007.

[30] E. Kalaiarasan and T. Palvannan, 'Journal of the taiwan institute of chemical engineers removal of phenols from acidic environment by horseradish peroxidase (hrp): aqueous thermostabilization of hrp by polysaccharide additives', *Journal of the Taiwan Institute of Chemical Engineers*, vol. 45, no. 2, pp. 625–634, 2014.

[31] R. Xu, Y. Si, F. Li and B. Zhang, 'Enzymatic removal of paracetamol from aqueous phase : horseradish peroxidase immobilized on nanofibrous membranes', *Environmental Science and Pollution Research*, vol. 22, pp. 3838–3846, 2015.

[32] A. M. Klibanov and E. D. Morris, 'Horseradish peroxidase for the removal of carcinogenic aromatic amines from water', *Enzyme and Microbial Technology*, vol. 3, pp. 119–122, 1981.

[33] C. Chen, X. Hong, T. Xu, A. Chen, L. Lu and Y. Gao, 'Hydrogen peroxide biosensor based on the immobilization of horseradish peroxidase onto a poly (aniline- co - n -methylthionine) film', *Synthetic Metals*, vol. 212, pp. 123–130, 2016.

[34] *Novozymes*, `http://www.novozymes.com/en/Pages/default.aspx`.

[35] A. Sassolas, L. J. Blum and B. D. Leca-bouvier, 'Immobilization strategies to develop enzymatic biosensors', *Biotechnology Advances*, vol. 30, pp. 489–511, 2012.

[36] Y. Jiang, W. Tang, J. Gao, L. Zhou and Y. He, 'Immobilization of horseradish peroxidase in phospholipid-templated titania and its applications in phenolic compounds and dye removal', *Enzyme and Microbial Technology*, vol. 55, pp. 1–6, 2014.

[37] P. Adlercreutz, 'Immobilisation and application of lipase in organic media', *Chemical Society Reviews*, vol. 42, no. 15, pp. 6406–6436, 2013.

[38] A. E. Danks, S. R. Hall and Z Schnepp, 'The evolution of 'sol-gel' chemistry as a technique for materials synthesis', *Royal Society of Chemistry - Materials Horizons*, 2015.

[39] A. M. A. Fidalgo, 'Síntese de materiais de porosidade controlada pelo processo sol-gel: dos xerogéis aos aerogéis de sílica', PhD thesis, Instituto Superior Técnico da Universidade Técnica de Lisboa, 2003.

[40] J. M. Deitzel, J Kleinmeyer, D Harris and N. C. B. Tan, 'The effect of processing variables on the morphology of electrospun nanofibers and textiles', *Polymer*, vol. 42, pp. 261–272, 2001.

[41] O. G. A. Lofgreen Jennifer E., 'Controlling morphology and porosity to improve performance of molecularly imprinted sol-gel silica', *Chemical Society Review*, vol. 43, p. 911, 2014.

[42] F. H. Dickey, 'Specific adsorption', vol. 491, no. 3, 1955.

[43] S. Braun, S. Rappoport, R. Zusman, D. Avnir and M. Ottolenghi, 'Biochemically active sol-gel glasses : the trapping of enzymes', *Materials Letters*, vol. 10, no. I, pp. 1–5, 1990.

[44] E. Biró, D. Budugan, A. Todea, F. Péter, S. Klébert and T. Feczkó, 'Recyclable solid-phase biocatalyst with improved stability by sol-gel entrapment of b-d-galactosidase', *Journal of Molecular Catalysis B: Enzymatic*, vol. 123, pp. 81–90, 2016.

[45] A. K. Patel, R. R. Singhania and A. Pandey, 'Novel enzymatic processes applied to the food industry', *Current Opinion in Food Science*, vol. 7, pp. 64–72, 2016.

[46] Y. Liu, C. Jiang, S. Li and Q. Hu, 'Composite vascular scaffold combining electrospun fi bers and physically-crosslinked hydrogel with copper wire-induced grooves structure', *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 61, pp. 12–25, 2016.

[47] V. B. Kandimalla, V. S. Tripathi and H. Ju, 'Immobilization of biomolecules in sol – gels : biological and analytical applications immobilization of biomolecules in sol – gels :' *Critical Reviews in Analytical Chemistry*, vol. 36, no. 2, pp. 37–41, 2006.

[48] E. Sotelo-gonzalez, A. M. Coto-garcia, M. T. Fernandez-argüelles, J. M. Costa-fernandez and A. Sanz-medel, 'Immobilization of phosphorescent quantum dots in a sol – gel matrix for acetone sensing', *Sensors and Actuators B : Chemical*, vol. 174, pp. 102–108, 2012.

[49] R. K. Satvekar, S. S. Rohiwal and A. V. Raut, 'A silica-dextran nanocomposite as a novel matrix for immobilization of horseradish peroxidase , and its application to sensing hydrogen peroxide', *Microchimica Acta*, vol. 181, pp. 71–77, 2014.

[50] I. Mazurenko, W. Ghach, G.-w. Kohring and C. Despas, 'Bioelectrochemistry immobilization of membrane-bounded (s) -mandelate dehydrogenase in sol – gel matrix for electroenzymatic synthesis', *Bioelectrochemistry*, vol. 104, pp. 65–70, 2015.

[51] N. A. Mohidem and H. Mat, 'The catalytic activity of laccase immobilized in sol-gel silica', *Journal of Applied Sciences*, vol. 9, no. 17, pp. 3141–3145, 2009.

[52] R. K. Das and M Das, 'Catalytic activity of acid and base with different concentration on sol-gel kinetics of silica by ultrasonic method', *Ultrasonics Sonochemistry*, vol. 26, pp. 210–217, 2015.

[53] M. K. Potdar, G. F. Kelso, L. Schwarz, C. Zhang and M. T. W. Hearn, 'Recent developments in chemical synthesis with biocatalysts in ionic liquids', *Molecules*, vol. 20, pp. 16 788–16 816, 2015.

[54] P. A. Thomas and B. B. Marvey, 'Room temperature ionic liquids as green solvent alternatives in the metathesis of', *Molecules*, vol. 21, no. 2, pp. 1–16, 2016.

[55] J. P. Tafur, F. Santos and A. J. F. Romero, 'Influence of the ionic liquid type on the gel polymer electrolytes properties', *Membranes*, vol. 5, pp. 752–771, 2015.

[56] F. V. Rantwijk and R. A. Sheldon, 'Biocatalysis in ionic liquids', *Chemical Reviews*, vol. 107, pp. 2757–2785, 2007.

[57] K. D. Weaver, J. Kim, J. Sun, R Macfarlane and G. D. Elliott, 'Cyto-toxicity and biocompatibility of a family of choline phosphate ionic liquids designed for pharmaceutical applications †', *Green Chemistry*, vol. 12, pp. 507–513, 2010.

[58] K. Fujita, D. R. Macfarlane, M. Forsyth, M. Yoshizawa-fujita, K. Murata, N. Nakamura and H. Ohno, 'Solubility and stability of cytochrome c in hydrated ionic liquids : effect of oxo acid residues and kosmotropicity', *Biomacromolecules*, vol. 8, pp. 2080–2086, 2007.

[59] K. Fujita, R Macfarlane and M. Forsyth, 'Protein solubilising and stabilising ionic liquids', *Chemical Communications*, vol. 70, pp. 4804–4806, 2005.

[60] R Vijayaraghavan, B. C. Thompson, D. R. Macfarlane, R. Kumar, M Surianarayanan, S Aishwarya and P. K. Sehgal, 'Biocompatibility of choline salts as crosslinking agents for collagen based biomaterials w', *Chemical Communications*, vol. 46, pp. 294–296, 2010.

[61] K. Fujita, K. Murata, M. Masuda, N. Nakamura and H. Ohno, 'Ionic liquids designed for advanced applications in bioelectrochemistry', *Royal Society of Chemistry*, vol. 2, pp. 4018–4030, 2012.

[62] K. D. Weaver, R. M. Vrikkis, M. P. V. Vorst, J. Trullinger, R. Vijayaraghavan, D. M. Foureau, I. H. Mckillop, D. R. Macfarlane, K Krueger and G. D. Elliott, 'Structure and function of proteins in hydrated choline dihydrogen phosphate ionic liquid w', *Phys. Chem. Chem. Phys.*, vol. 14, pp. 790–801, 2012.

[63] P. Vidinha, N. M. T. Lourenço, C. Pinheiro, A. R. Brás, T. Carvalho, T. Santos-Silva, A. Mukhopadhyay, M. J. Romão, J. Parola, M. Dionisio, J. M. S. Cabral, C. A. M. Afonso and S. Barreiros, 'Ion jelly : a tailor-made conducting material for smart electrochemical devices', *Chemical Communications*, pp. 5842–5844, 2008.

[64] H. He, J. Wang, Q. Gao, M. Chang, Z. Ren, X. Zhang, X. Li, W. Weng and G. Han, 'Colloids and surfaces b : biointerfaces ag-silica composite nanotube with controlled wall structures for biomedical applications', *Colloids and Surfaces B: Biointerfaces*, vol. 111, pp. 693–698, 2013.

[65] W.-e. Teo, R. Inai and S. Ramakrishna, 'Technological advances in electrospinning of nanofibers', *Science and Tecnhonology of Advanced Materials*, vol. 12, p. 19, 2011.

[66] A. Haider, S. Haider and I.-k. Kang, 'Comprehensive review summarizing effect of electrospinning parameters and potencial applications of nanofibers in biomedical and biotechnology', *Arabian Journal of Chemistry*, 2015.

[67] A. Repanas, S. Andriopoulou and B. Glasmacher, 'The significance of electrospinning as a method to create fibrous scaffolds for biomedical engineering and drug delivery applications', *Journal of Drug Delivery Science and Technology*, vol. 31, pp. 137–146, 2016.

[68] W. Sigmund, J. Yuh, H. Park, V. Maneeratana, G. Pyrgiotakis, A. Daga, J. Taylor and J. C. Nino, 'Processing and structure relationships in electrospinning of ceramic fiber systems', *Journal of the American Ceramic Society*, vol. 89, no. 2, pp. 395–407, 2006.

[69] S. Zhang and Y. Tang, 'A review on preparation and applications of silver-containing nanofibers', *Nanoscale Research Letters*, vol. 80, no. 11, pp. 1–8, 2016.

[70] H. Wu, W. Pan, D. Lin and H. Li, 'Electrospinning of ceramic nanofibers : fabrication , assembly and applications', *Journal of Advanced Ceramics*, vol. 1, no. 1, pp. 2–23, 2012.

[71] S. Nagamine, T. Matsumoto, Y. Hikima and M. Ohshima, 'Fabrication of porous carbon nanofibers by phosphate-assisted carbonization of electrospun poly (vinyl alcohol) nanofibers', *Materials Research Bulletin*, 2016.

[72] K. Mondal, A. Ali, S. Srivastava, B. D. Malhotra and A. Sharma, 'Electrospun functional micro/nanochannels embedded in porous carbon electrodes for microfluidic biosensing', *Sensors & Actuators: B. Chemical*, vol. 229, pp. 82–91, 2016.

[73] B. Ma, J. Xie, J. Jiang, F. D. Shuler and D. E. Bartlett, 'Rational design of nanofiber scaffolds for orthopedic tissue repair and regeneration', *Nanomedicine*, vol. 8, no. 9, pp. 1459–1481, 2013.

[74] H. M. Khanlou, B. C. Ang, S. Talebian, M. M. Barzani, M. Silakhori and H. Fauzi, 'Multi-response analysis in the processing of poly (methyl methacrylate) nanofibres membrane by electrospinning based on response surface methodology : fibre diameter and bead formation', *Measurement*, vol. 65, pp. 193–206, 2015.

[75] J. Zhao, N. Si, L. Xu, X. Tang, Y. Song and Z. Sun, 'Experimental and theoretical study on the electrospinning nanoporous fi bers process', *Materials Chemistry and Physics*, vol. 170, pp. 294–302, 2016.

[76] P. Lu and B. Ding, 'Applications of electrospun fibers', *Recent Patents on Nanotechnology*, vol. 2, pp. 169–182, 2008.

[77] I. S. Chronakis, 'Novel nanocomposites and nanoceramics based on polymer nanofibers using electrospinning process — a review', *Journal of Materials Processing Technology*, vol. 167, pp. 283–293, 2005.

[78] A. Frenot and I. S. Chronakis, 'Polymer nanofibers assembled by electrospinning', *Current Opinion in Colloid and Interface Science*, vol. 8, pp. 64–75, 2003.

[79] P. Jochems, Y. Satyawali and W. Dejonghe, 'Enzyme immobilization on/in polymeric membranes : status, challenges and perspectives in biocatalytic membrane reactors (bmrs)', *Green Chemistry*, vol. 13, pp. 1609–1623, 2011.

[80] L. Giorno and E. Drioli, 'Biocatalytic membrane reactors: applications and perspectives', *Trends in Biotechnology*, vol. 18, pp. 339–349, 2000.

[81] H.-w. Tong, B. R. Mutlu, L. P. Wackett and A. Aksan, 'Manufacturing of bioreactive nanofibers for bioremediation', *Biotechnology and Bioengineering*, vol. 111, no. 8, pp. 1483–1493, 2014.

[82] P. Kortesuo, M. Ahola, M. Kangas and A. Yli-urpo, 'In vitro release of dexmedetomidine from silica xerogel monoliths : effect of sol-gel synthesis parameters', *International Journal of Pharmaceutics*, vol. 221, pp. 107–114, 2001.

[83] A. Kierys, R. Kasperek, P. Krasucka and J. Goworek, 'Encapsulation of diclofenac sodium within polymer beads by silica species via vapour-phase synthesis', *Colloids and Surfaces B: Biointerfaces*, vol. 142, pp. 30–37, 2016.

[84] Y. Bao, C. Shi, T. Wang, X. Li and J. Ma, 'Recent progress in hollow silica: template synthesis, morphologies and applications', *Microporous and Mesoporous Materials*, vol. 227, pp. 121–136, 2016.

[85] L. T. H. Nguyen, S. Chen, N. K. Elumalai, M. P. Prabhakaran, Y. Zong, C. Vijila, S. I. Allakhverdiev and S. Ramakrishna, 'Biological , chemical , and electronic applications of nanofibers', *Macromolecular Materials and Engineering*, vol. 298, no. 8, pp. 822–867, 2013. DOI: 10.1002/mame.201200143.

[86] J. Wang, P. Zhou, A. Obata, J. R. Jones and T. Kasuga, 'Preparation of cotton-wool-like poly (lactic acid) -based composites consisting of core-shell-type fibers', *Materials*, vol. 8, pp. 7979–7987, 2015.

[87] C. Nanofibers, 'Ceramic nanofibers and nanotubes', *The American Ceramic Society*, vol. 1869, no. 6, pp. 1861–1869, 2006.

[88] A. L. Yarin, E Zussman, J. H. Wendorff and A Greiner, 'Material encapsulation and transport in core – shell micro / nanofibers , polymer and carbon nanotubes and micro / nanochannels', *Journal of Materials Chemistry*, vol. 17, pp. 2585–2599, 2007.

[89] A. L. Yarin, 'Coaxial electrospinning and emulsion electrospinning of core – shell fibers', *Polymers Advanced Technology*, vol. 22, pp. 310–317, 2011.

[90] R. Obert and B. C. Dave, 'Enzymatic conversion of carbon dioxide to methanol : enhanced methanol production in silica sol - gel matrices', *Journal of the American Chemical Society*, vol. 51, pp. 12 192–12 193, 1999.

[91] T. Pirzada, S. A. Arvidson, C. D. Saquing, S. S. Shah and S. A. Khan, 'Hybrid silica - pva nanofibers via sol - gel electrospinning', *Langmuir*, vol. 28, pp. 5834–5844, 2012.

[92] D. A. Oriero, A. T. Weakley and D. E. Aston, 'Rheological and micro-raman time-series characterization of enzyme sol – gel solution toward morphological control of electrospun fibers', *Science and Tecnhology of Advanced Materials*, vol. 13, no. 2, pp. 1–10, 2012.

[93] N. Shankhwar and A Srinivasan, 'Evaluation of sol – gel based magnetic 45s5 bioglass and bioglass – ceramics containing iron oxide', *Materials Science & Engineering C*, vol. 62, pp. 190–196, 2016.

[94] V. C. Costa, H. S. Costa, W. L. Vasconcelos, M. D. M. Pereira, R. L. Oréfice and H. S. Mansur, 'Preparation of hybrid biomaterials for bone tissue engineering', *Materials Research*, vol. 10, no. 1, pp. 21–26, 2007.

[95] D. C. Joy, 'Second best no more', *Nature Materials*, vol. 8, no. 10, pp. 776–777, 2009.

[96] Y Zhu, H Inada, K Nakamura and J Wall, 'Imaging single atoms using secondary electrons with an aberration-corrected electron microscope', *Nature Materials*, vol. 8, no. 10, pp. 808–812, 2009.

[97] U. Schubert, 'Chemistry and fundamentals of the sol-gel process', in *The Sol-Gel Handbook: Synthesis, Characterization, and Applications*, D. Levy and M. Zayat, Eds., First Edit, Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA, 2015, pp. 1–27.

[98] B Sun, Y. Z. Long, H. D. Zhang, M. M. Li, J. L. Duvail, X. Y. Jiang and H. L. Yin, 'Progress in polymer science advances in three-dimensional nanofibrous macrostructures via electrospinning', *Progress in Plymer Science*, vol. 39, pp. 862–890, 2014.

[99] C. R. Wittmer, A. Hébraud, S. Nedjari and G. Schlatter, 'Well-organized 3d nanofibrous composite constructs using cooperative effects between electrospinning and electrospraying', *Polymer*, vol. 55, no. 22, pp. 5781–5787, 2014.

[100] M.A.Fardad, 'Catalysts and the structure of sio 2 sol-gel films', *Journal of Materials Science*, vol. 5, pp. 1835–1841, 2000.

[101] M. Ramezani, M. R. Vaezi and A. Kazemzadeh, *The influence of the hydrophobic agent, catalyst, solvent and water content on the wetting properties of the silica films prepared by one-step sol-gel method.* Elsevier B.V., 2015, pp. 99–106.

[102] P. Innocenzi, 'Infrared spectroscopy of sol – gel derived silica-based films : a spectra-microstructure overview', *Journal of non-crystalline solids*, vol. 316, pp. 309–319, 2003.

[103] *Aerogel*, `http://www.aerogel.org/wpcontent/uploads/2009/03/sol-gel-alkoxide-fit.gif`.

[104] C. C. Thong, D. C. L. Teo and C. K. Ng, 'Application of polyvinyl alcohol (pva) in cement-based composite materials : a review of its engineering properties and microstructure behavior', *Construction and Building Materials*, vol. 107, pp. 172–180, 2016.

[105] Y. W. Mpharm, Q. Zhao, N. Han, L. B. Mpharm, J. L. Mpharm, J. L. Mpharm, E. C. Mpharm, L. H. Mpharm, Q. Zhang, T. Jiang and S. Wang, 'Mesoporous silica nanoparticles in drug delivery and biomedical applications', *Nanomedicine: Nanotechnology, Biology, and Medicine*, vol. 11, pp. 313–327, 2015.

[106] K. P. Ananth, A. J. Nathanael, S. P. Jose, T. H. Oh and A. M. Ballamurugan, 'A novel silica nanotube reinforced ionic incorporated hydroxyapatite composite coating on polypyrrole coated 316l ss for implant application', *Materials Science & Engineering C*, vol. 59, pp. 1110–1124, 2016.